In-vitro Assessment of Cholinesterase Inhibitory, Antioxidant and Thrombolytic Activity of *Citrus macroptera* fruit extracts

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

Submitted by

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Dedicated to my mother, Shaila Salam, my family, my respected teachers and my friends, for their love and support…

Certificate by the Chairperson

This is to certify that the thesis entitled "In-vitro Assessment of Cholinesterase Inhibitory, Antioxidant and Thrombolytic Activity of *Citrus macroptera* fruit extracts" 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 Saiyara Hossain Reevu, ID: 2013-1-70-058, during the period 2016 of her research in the Department of Pharmacy, East West University.

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Certificate by the Supervisor

This is to certify that the thesis entitled "In-vitro Assessment of Cholinesterase Inhibitory, Antioxidant and Thrombolytic Activity of *Citrus macroptera* fruit extracts" 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 Saiyara Hossain Reevu, ID: 2013-1-70-058, during the period 2016 of her research in the Department of Pharmacy, East West University, under the supervision and guidance of me. The thesis has not formed the basis for the award of any other degree/diploma/fellowship or other similar title to any candidate of any university.

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Declaration by the Candidate

I, Saiyara Hossain Reevu, hereby declare that the dissertation entitled "In-vitro Assessment of Cholinesterase Inhibitory, Antioxidant and Thrombolytic Activity of *Citrus macroptera* fruit extracts" submitted by me to the Department of Pharmacy, East West University and in the partial fulfillment of the requirement for the award of the degree Bachelor of Pharmacy, work carried out by me during the period 2016 of my research in the Department of Pharmacy, East West University, under the supervision and guidance of Kushal Biswas, Lecturer, Department of Pharmacy, East West University. The thesis paper has not formed the basis for the award of any other degree/diploma/fellowship or other similar title to any candidate of any university.

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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, Kushal Biswas, Lecture, Department of Pharmacy, East West University who has been always optimistic and full of passion and ideas. His 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. His 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. Shamsun Nahar Khan, Associate Professor and Chairperson of Pharmacy Department; Dr. Sufia Islam, Associate Professor of Pharmacy Department; 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.

Thirdly, I am thanking our respected lab officers Sipra Biswas, Ruchira Chakrabarty, Ajoy Roy, and Shofiqul Islam for helping me by providing equipment and materials as well as their guidance I needed to fulfill the work.

Fourth, my special thanks Taposhi Sultana Mou, Farzana Khan Sristy, 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.

Finally, I am immensely grateful to my dear mother, Shaila Salam without whom I wouldn't be who I am now. Her love and faith in me, made me stronger and motivated me. It is my mother without whom I wouldn't have come this far. I also would like to express my heartfelt love to my grandparents, Abdus Salam Mollah and Shamsi Ara Salam, and my family for their continuous support and love.

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Abbreviation	Full Form
Ach	Acetyl Choline
AChE	Acetyl Cholinesterase
AD	Alzheimer's Disease
ATP	Adenosine Triphosphate
ATCI	Acetyl Thiocholine Iodide
BTCI	Butyryl Thiocholine Iodide
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	Millimolar
NaCl	Sodium Chloride
nm	Nanometer
STD	Standard Deviation
TCA	Trichloro acetic acid
UV	Ultraviolet

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, and the most predominant cause of dementia in the elderly. Traditionally *Citrus macroptera* (L.) has widely been used as antioxidant source. Our aim is to investigate the cholinesterase inhibitory activities, antioxidant properties as well as thrombolytic properties of the fruits pulp and peel extracts in the treatment of AD and clotting disorders. The crude methanol extract was prepared by cold extraction method and was assessed for acetyl cholinesterase and butyryl cholinesterase inhibitory activity by the Ellman method; their antioxidant properties were assessed by several assays including reducing power, scavenging of DPPH and hydroxyl radicals together with quantitative analysis of endogenous substances. Its thrombolytic potential activity is determined by clot lysis method. Quantitative analysis displayed higher contents of phenolics and flavonoids in the crude methanolic fraction. The results revealed that the ethyl acetate extract, possibly due to its phenolic compounds, exerts potential antioxidant and cholinesterase inhibitory activities which may be useful in the treatment of AD. But in case of thrombolytic property it seems not so potential at all.

1.1. Introduction

Alzheimer's disease (AD) is a progressive and irreversible brain disorder and the most common form or cause of dementia in which the memory and thinking skills and eventually the ability to carry out the simple tasks are destroyed slowly. Dementia refers to the loss of cognitive functioning, that is, thinking, remembering, reasoning and behavioral abilities to such an extent that it interferes with a person's daily life and activities. The symptoms of dementia gradually worsen over a number of years. Alzheimer's is the general term for memory loss and other intellectual abilities. In the early stages of Alzheimer's, memory loss is mild but with late stage individuals lose the ability to carry on a conversation and respond to their environment. (Alz.org, 2016)

Alzheimer's disease has been referred as a 'dual clinicopathological entity', typically centred on the presence of a progressive dementia that includes episodic memory impairment as a defining feature and involvement of other cognitive skills, and specific neuropathological changes that usually include intraneuronal and extracellular parenchymal lesions often accompanied by synaptic loss and vascular amyloid deposits. (Dubois, B. et. al, 2010)

Alzheimer's disease is named after Dr. Alois Alzheimer. In 1906, Dr. Alzheimer noticed changes in the brain tissue of a woman who had died of an unusual mental illness. Her symptoms included memory loss, language problems and unpredictable behavior. After she died, he examined her brain and found many abnormal clumps (now called amyloid plaques) and tangled bundles of fibers (now called neurofibrillary, or tau, tangles). These plaques and tangles in the brain are still considered some of the main features of Alzheimer's disease. Another feature is the loss of connections between nerve cells (neurons) in the brain. Neurons transmit messages between different parts of the brain, and from the brain to muscles and organs in the body. (Alz. org, 2016)

1.2. Causes of Alzheimer's Disease

The cause of Alzheimer's disease in most people is not fully understood yet by the scientists. Usually a genetic mutation is the cause in people with early-onset Alzheimer's. Late-onset Alzheimer's arises from a complex series of brain changes that occur over decades. The causes probably include a combination of genetic, environmental and lifestyle factors. The importance of any one of these factors in increasing or decreasing the risk of developing Alzheimer's may differ from person to person. (Alz.org, 2016)

Studies are being conducted by scientists to learn more about plaques, tangles, and other biological features of Alzheimer's disease. Researchers can see the development and spread of abnormal amyloid and tau proteins in the living brain, as well as changes in brain structure and function through the advances in brain imaging techniques. Scientists are also exploring the very earliest steps in the disease process by studying changes in the brain and body fluids that can be detected years before Alzheimer's symptoms appear. Findings from these studies will help in understanding the causes of Alzheimer's and make diagnosis easier.

In most people with Alzheimer's, symptoms first appear in their mid-60s. One of the great mysteries of this disease is why it largely strikes older adults. Research on normal brain aging is shedding light on this question. Scientists are learning how age-related changes in the brain may harm neurons and contribute to Alzheimer's damage which include atrophy (shrinking) of certain parts of the brain, inflammation, production of unstable molecules called free radicals, and mitochondrial dysfunction (a breakdown of energy production within a cell). (Alz.org, 2016)

1.3. Signs and Symptoms

One of the first signs of cognitive impairment related to Alzheimer's disease is typically memory loss. The symptoms vary from person to person. For many, decline in non-memory aspects of cognition, such as word-finding, vision/spatial issues, and impaired reasoning or judgment, may signal the very early stages of Alzheimer's disease.

The most common initial symptom is a gradually worsening ability to remember new information. This occurs because the first neurons to be damaged and destroyed are usually in brain regions involved in forming new memories. As neurons in other parts of the brain are damaged and destroyed, individuals experience other difficulties.

Just like the rest of our bodies, our brains change as we age. Most of us eventually notice some slowed thinking and occasional problems with remembering certain things. However, serious memory loss, confusion and other major changes in the way our minds work may be a sign that brain cells are failing.

The most common early symptom of Alzheimer's is difficulty remembering newly learned information because Alzheimer's changes typically begin in the part of the brain that affects learning. As Alzheimer's advances through the brain it leads to increasingly severe symptoms includes disorientation, changes in mood and behavior, deepening confusion about events, time and place, unfounded suspicions about family, friends and professional caregivers, more serious memory loss and behavior changes and difficulty in speaking, swallowing and walking.

a. Memory loss interfering with daily activities

Memory loss is one of the most common signs of Alzheimer's, especially forgetting recently learned information. Others include forgetting important dates or events, asking for the same information over and over, increasingly needing to rely on memory aids (e.g., reminder notes or electronic devices) or family members for things they used to handle on their own.

b. Difficulty in solving problems

Some people may experience changes in their ability to develop and follow a plan or work with numbers. They may have difficulty concentrating and take much longer to do things than they did before.

c. Problems in completing familiar tasks

People with Alzheimer's often find it hard to complete daily tasks. Sometimes, people may have trouble driving to a familiar location, managing a budget at work or remembering the rules of a favorite game.

d. Confusion with time and place

People with Alzheimer's can lose track of dates, seasons and the passage of time. They may have trouble understanding something if it is not happening immediately. Sometimes they may forget where they are or how they got there.

e. Vision Problems

For some people, having problems with visual images is a sign of Alzheimer's. They may have difficulty reading, judging distance and determining color or contrast, which may cause problems with driving.

f. Difficulty in writing and speaking

People with Alzheimer's may have trouble following or joining a conversation. They may stop in the middle of a conversation and have no idea how to continue or they may repeat themselves. They may struggle with vocabulary, have problems finding the right word or call things by the wrong name.

g. Misplacing and forgetting things

A person with Alzheimer's disease may put things in unusual places. They may lose things and be unable to go back over their steps to find them again.

h. Difficulty in judgment

People with Alzheimer's may experience changes in judgment or decision-making. For example, they may use poor judgment when dealing with money, giving large amounts to telemarketers. They may pay less attention to grooming or keeping themselves clean.

i. Withdrawal from work or social activities

A person with Alzheimer's may start to remove themselves from hobbies, social activities, work projects or sports. They may have trouble keeping up with a favorite sports team or remembering how to complete a favorite hobby. They may also avoid being social because of the changes they have experienced.

j. Mood and personality change

The mood and personalities of people with Alzheimer's can change. They can become confused, suspicious, depressed, fearful or anxious. They may be easily upset at home, at work, with friends or in places where they are out of their comfort zone.

1.4. Stages of Alzheimer's Disease

Overview of disease progression

The rate at which the disease advances varies but the symptoms of Alzheimer's disease may worsen over time. On average, a person with Alzheimer's lives four to eight years after diagnosis, but can live as long as 20 years, depending on other factors.

Changes in the brain related to Alzheimer's begin years before any signs of the disease. This time period, which can last for years, is referred to as preclinical Alzheimer's disease.

The stages are separated into three different categories: Mild Alzheimer's disease, Moderate Alzheimer's disease and Severe Alzheimer's disease.

Mild Alzheimer's disease (early-stage)

In the early stages of Alzheimer's, a person may function independently. He or she may still drive, work and be part of social activities. Despite this, the person may feel as if he or she is having memory lapses, such as forgetting familiar words or the location of everyday objects.

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

- Problems coming up with the right word or name
- Trouble remembering names when introduced to new people
- Having greater difficulty performing tasks in social or work settings
- Forgetting material that one has just read
- Losing or misplacing a valuable object
- Increasing trouble with planning or organizing

Moderate Alzheimer's Disease (middle-stage)

In this stage, damage occurs in areas of the brain that control language, reasoning, sensory processing, and conscious thought. Memory loss and confusion grow worse, and people begin to have problems recognizing family and friends. They may be unable to learn new things, carry out multistep tasks such as getting dressed, or cope with new situations. In addition, people at this stage may have hallucinations, delusions and paranoia and may behave impulsively.

Moderate Alzheimer's is typically the longest stage and can last for many years. As the disease progresses, the person with Alzheimer's will require a greater level of care. The person with Alzheimer's can be noticed with confusing words, getting frustrated or angry, or acting in unexpected ways, such as refusing to bathe. Damage to nerve cells in the brain can make it difficult to express thoughts and perform routine tasks.
At this point, symptoms will be noticeable to others and may include:

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

Severe Alzheimer's Disease (late stage)

Ultimately, plaques and tangles spread throughout the brain, and brain tissue shrinks significantly. People with severe Alzheimer's cannot communicate and are completely dependent on others for their care. Near the end, the person may be in bed most or all of the time as the body shuts down.

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

At this stage, individuals may:

- Require full-time, around-the-clock assistance with daily personal care
- Lose awareness of recent experiences as well as of their surroundings
- Require high levels of assistance with daily activities and personal care
- Experience changes in physical abilities, including the ability to walk, sit and, eventually, swallow
- Have increasing difficulty communicating
- Become vulnerable to infections, especially pneumonia

1.5. Current Trend

- Worldwide, nearly 44 million people have Alzheimer's or a related dementia.
- Only 1-in-4 people with Alzheimer's disease have been diagnosed.
- Alzheimer's and dementia is most common in Western Europe (North America is close behind).
- Alzheimer's is least prevalent in Sub-Saharan Africa.
- Alzheimer's and other dementias are the top cause for disabilities in later life.

The number of Americans with Alzheimer's disease is growing and growing fast.

- An estimated 5.4 million Americans of all ages have Alzheimer's disease in 2016.
- Of the 5.4 million Americans with Alzheimer's, an estimated 5.2 million people are age 65 and older, and approximately 200,000 individuals are under age 65 (younger-onset Alzheimer's).

- One in nine people age 65 and older has Alzheimer's disease.
- By mid-century, someone in the United States will develop the disease every 33 seconds. (Alz.org, 2016)

The Cost of Alzheimer's Care

- The cost of caring for Alzheimer's patients in the U.S. is estimated to be $236 billion in 2016. (Alz.org, 2016)
- The global cost of Alzheimer's and dementia is estimated to be $605 billion, which is equivalent to 1% of the entire world's gross domestic product. (Alzheimers.net)

1.6. Treatment of Alzheimer's Disease

Different Hypothesis for the Pathogenesis of Alzheimer's disease:

The pathogenesis of Alzheimer's disease is highly complex. While several pathologies characterize this disease, amyloid plaques, composed of the β-amyloid peptide are hallmark neuropathological lesions in Alzheimer's disease brain. Indeed, a wealth of evidence suggests that β-amyloid is central to the pathophysiology of AD and is likely to play an early role in this intractable neurodegenerative disorder. Many molecular lesions have been detected in Alzheimer's disease, but the overarching theme to emerge from the data is that an accumulation of misfolded proteins in the aging brain results in oxidative and inflammatory damage, which in turn leads to energy failure and synaptic dysfunction. There are several hypothesis are established for the pathogenesis of AD. They are:

a. β-Amyloid cascade hypothesis,

b. Cholinergic hypothesis,

c. Oxidative stress hypothesis,

d. Tau hypothesis,

e. Inflammatory hypothesis.

1.6.1. β-Amyloid Cascade Hypothesis:

Generation of Aβ from APP requires two proteolytic processes by several different proteases. The amyloidogenic pathway results in production of intact Aβ, whereas the

non-amyloidogenic pathway precludes intact Aβ-formation. In the amyloidogenic pathway, APP is first cleaved at the β-secratase site by β-site cleaving enzyme (BACE), generating a soluble extracellular fragment of APP (sAPPβ) and a membrane-bound 99 amino acid residue C-terminal fragment (C99). C99 is further processed by cleavage at the γ-secretase site, located within the APP trans-membrane domain, to generate Aβ-peptides of different lengths of the two main variants formed; Aβ1-40 is the most abundant whereas Aβ1-42 is produced to a lesser. The amyloid cascade hypothesis has been one of the leading theories in the pathogenic events causing AD. This hypothesis postulates a causative role for Aβ deposition in the pathogenesis of AD, and the NFTs, inflammatory response, cell loss, vascular damage and dementia follow as a direct result of this deposition. Evidence that Aβ play a casual role in the development of AD came from the following studies. First, mutations in APP, PS1 and PS2 genes that are linked to the early onset forms of AD increase the production of total Aβ or specifically increase the relative amount of Aβ1-42. Second, individuals with Down's syndrome overproduce Aβ and develop AD-like dementia and neuropathology. Third, the levels of deposited Aβ correlate with cognitive decline and severity of the disease in both AD patients and transgenic mice. Fourth, fibrillar Aβ has been shown to be neurotoxic *in vitro* and is able to mediate neurotoxic effects, inflammatory responses and abnormal tau phosphorylation. Moreover, several reports suggest a casual link between Aβ and impaired neuronal function and cognitive decline. Recently, attention has been focused on which form (monomers, oligomers, protofibrils or fibrils) of amyloid species has the most deleterious effects. Several studies have suggested that the oligomeric and protofibrillar forms of amyloid as the most toxic. Even if the amyloid cascade hypothesis is convincing, it does not fully explain the role of tangles and/or inflammatory response. One argument against the amyloid cascade hypothesis is that the APP/PSI double transgenic mice only develop plaques pathology, but not the NFTs in the brain. Recently, amyloid deposition was shown to precede tangle formation in a triple transgenic mouse model overexpressing mutant APP and mutant tau on a PSI mutation Knock-in background.

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precede tangle formation in a triple transgenic mouse model overexpressing mutant APP and mutant tau on a PSI mutation Knock-in background.

1.6.2. Cholinergic Hypothesis:

Acetylcholine is an important neurotransmitter in brain regions involving memory. As expected, loss of cholinergic activity correlates with cognitive impairment. A variety of studies in humans indicate that basal forebrain and rostral forebrain cholinergic pathways including converging projections to the thalamus serve important functional roles in conscious awareness, attention, working memory and a number of additional mnemonic processes.

For more than 20 years, studies of the brains of those with advanced age and Alzheimer's disease (AD) have consistently found damage or abnormalities in these pathways (particularly basal forebrain projections) that appeared to correlate well with the level of cognitive decline. As a result, the so-called "cholinergic hypothesis" was developed, which essentially states that a loss of cholinergic function in the central nervous system contributes significantly to the cognitive decline associated with advanced age and AD. In AD, cholinergic abnormalities are the most prominent of neurotransmitter changes, primarily because of the reduced activity of choline acetyl transferase (an enzyme involved in acetylcholine synthesis). By late-stage AD, the number of cholinergic neurons are markedly reduced, particularly in the basal forebrain. One of the most prominent features observed in AD patients is a deficiency of acetylcholine (ACh), a neurotransmitter found in the synapses of the cerebral cortex. The cholinesterase enzyme exists in two different forms in humans, AChE and butyryl cholinesterase (BuChE). AChE is the main cholinesterase in the CNS, while BuChE, originated from glial cells, is more common in serum. Decreased AChE activity and stable or increased BuChE activities were detected in the brains of AD. Most of the neocortical AChE activity in AD brain was found associated with NPs, in which it colocalized with Aβ deposits including both the diffuse amyloid deposits and the mature NPs. Further studies showed that AChE promoted the aggregation of Aβ peptides and accelerated the formation of amyloid plaque, suggesting that AChE may play a pathogenic role in AD by influencing Aβ processing. It has also been shown that Aβ which aggregates with AChE is more toxic to cells compared to aggregates of Aβ alone.

1.6.3. Oxidative stress hypothesis:

Oxidation can include the combination of a substance with oxygen or free radical damage. The oxidative process of adding an oxygen molecule to a protein can be a normal part of cellular function or may be aberrant, with a resulting change in the protein's form and ability to function properly. Free radical damage occurs when an oxygen or nitrogen molecule containing an unpaired extra electron (termed species) reacts with other molecules to achieve a stable configuration. During this process a high-energy election is thrown off (termed a free radical) that can cause cellular and molecular damage. Either of these oxidative processes can cause oxidative stress with resulting cellular damage from oxidase. The central nervous system (CNS) is especially vulnerable to free radical damage as a result of the brain's high oxygen consumption rate, its abundant lipid content and the relative paucity of antioxidant enzymes compared with other tissues. AD brain is under extensive oxidative stress as manifested by lipid peroxidation, protein oxidation and DNA oxidation.[42] Aβ might be the central to the pathogenesis of AD. Aβ has been shown to induce protein oxidation and lipid peroxidation *in vitro* and *in vivo*. Many studies have indicated that Aβ-induced oxidative stress is involved in the pathogenesis of AD.

Lipid peroxidation is an important mechanism of neurodegeneration in AD brain. Many studies have shown increased lipid peroxidation in several regions of AD brain, where the histopathologic alterations are very noticeable. It has been shown that there is a strong regional correlation between the thiobarbituric acid reactive substances (TBARS), one indicator of lipid peroxidation, antioxidant enzymes, the presence of NPs and NFT in AD brain. Aβ is widely reported to cause lipid peroxidation in brain cell membranes in a manner that is inhibited by free radical antioxidants.

Aβ leads to an increased level of 4-hydroxy-2-nonenal (4-FINE), one of the major products of lipid peroxidation, in hippocampal and cortical neuronal cells. Increased 4-FINE was found in AD brain and it was proven to be toxic to hippocampal neuronal cells. 4-HNE can also increase the vulnerability of cultured hippocampal neurons to excitotoxicity, as well as an alteration in multiple cellular functions including glucose or glutamate transport.

1.6.4. Tau Hypothesis

Neurofibrillary tangles, which are filamentous inclusions in pyramidal neurons, occur in Alzheimer's disease and other neurodegenerative disorders termed tau hypothesis.

The number of neurofibrillary tangles is a pathologic marker of the severity of Alzheimer's disease. The major component of the tangles is an abnormally hyper phosphorylated and aggregated form of tau. Normally an abundant soluble protein in axons, tau promotes assembly and stability of microtubules and vesicle transport. Hyper phosphorylated tau is insoluble, lacks affinity for microtubules and self-associates into paired helical filament structures. Enzymes that add and those that remove phosphate residues regulate the extent of tau phosphorylation. Like Aβ oligomers, intermediate aggregates of abnormal tau molecules are cytotoxic and impair cognition. Insoluble helical filaments may be inert, however, since decreases in axonal transport and neuron number are independent of the burden of neurofibrillary tangles. These helical filaments sequester toxic intermediate tau species, a process that may be protective. More than 30 mutations of *Tau* on chromosome 17 have been detected in front temporal dementia with Parkinsonism. By contrast, *Tau* mutations do not occur in Alzheimer's disease, and the extent of neuron loss is out of proportion to the number of neurofibrillary tangles. Nevertheless, increased levels of phosphorylated and total tau in the cerebrospinal fluid correlate with reductions in scores on cognitive examinations. Elevated levels of phosphotau amino acids T181, T231, and total tau in the cerebrospinal fluid together constitute a biomarker test with good accuracy for predicting incipient Alzheimer's disease in patients with mild cognitive impairment. Experimental evidence indicates that Aβ accumulation precedes and drives tau aggregation. Moreover, Aβ-induced degeneration of cultured neurons and cognitive deficits in mice with an Alzheimer's disease–like illness require the presence of endogenous tau. Increased oxidative stress, the impaired protein-folding function of the endoplasmic reticulum and deficient proteasome-mediated and autophagic-mediated clearance of damaged proteins-

a. All of which are also associated with aging,

b. Accelerate the accumulation of amyloid and tau proteins in Alzheimer's disease.

1.6.5. Inflammatory Hypothesis:

Neurons are not the only brain cells affected in AD. Microglia, 1 of 3 glial cell types (along with astrocytes and oligodendrocytes) in the CNS, is involved in immune and inflammatory responses to injury or infection within the brain. Several lines of evidence have proposed that inflammation may play a significant role in the pathogenesis of AD. According to the "inflammation hypothesis", the deposition of amyloid in AD brain brings about activation of microglia and astrocytes, initiating a pro-inflammatory cascade that results in the release of potentially neurotoxic substances, cytokines and other related compounds, bringing about degenerative changes in neurons. Reactive astrocytes and activated microglia are often found in and around amyloid plaques in the brains of AD patients and APP transgenic mice.

Other hypotheses:

Herpes simplex virus type 1 has been proposed to play a causative role in people carrying the susceptible versions of the apoE gene. Some have hypothesized that dietary copper may play a causal role.

Another hypothesis asserts that the disease may be caused by age related myelin breakdown in the brain. Iron released during myelin breakdown is hypothesised to cause further damage. Homeostatic myelin repair processes contribute to the development of proteinaceous deposits such as beta-amyloid and tau.

1.7. Plants used for the treatment of Alzheimer's Disease :

The drugs available for the treatment of the disease have limited effectiveness, for such, no cure for Alzheimer's exists. It is believed that the number of cases will be dramatically reduced in the next 50 years because of the therapeutic intervention that could postpone the onset or progression of Alzheimer's disease. For the development of drugs, medicinal plants, for example, ayurvedic plants have been the single most productive source of leads. Many scientific studies have described the use of various medicinal plants and their constituents for treatment of Alzheimer's disease. Phytochemical studies of the different parts of the plants have shown the presence of many valuable compounds, such as lignans, flavonoids, tannins, polyphenols, triterpenes, sterols, and alkaloids, that show a wide spectrum of pharmacological activities, including anti-inflammatory, anti-cholinesterase, hypolipidemic, and

antioxidant effects though the exact mechanism of their action is still not clear. (Rao, R. V., et. al, 2012)

a. *Withania somnifera* (Ashwagandha)

Ashwagandha belonging to the Solanaceae family is used extensively as an aphrodisiac, rejuvenative, adaptogen and helps the body adapt to stress. The root part is used widely. It is thought that it has an ability to support the immune system along with antioxidant activity, free radical scavenging activity. It also works as a stimulant and gives a calming effect and so can be used in patients with Alzheimer's disease.

b. *Curcuma longa L.* (Turmeric)

Turmeric is has been commonly used as a dietary spice in Southeast Asian countries where a 4.4 fold lower incidence of AD has been shown according to an epidemiologic study. Other studies show that it reduces the risk of AD by its non-steroidal anti-inflammatory property. It also reduces oxidative damage and reversed the amyloid pathology in an AD transgenic mouse. It has been shown that, it reduces the amount of plaque deposition when fed to aged mice with advanced plaque deposits which is similar to AD. It belongs to the Zingiberaceae family and used as a source of Curcumin which is an orange-yellow component of turmeric or curry powder. This herb helps to keep the mind balanced upon regular consumption. (Singhal, A.K., et. al, 2012)

c. *Bacopa monnieri* (Brahmi)

Bacopamonnieri which is also known as Brahmi is used as a therapeutic agent for insomnia, epilepsy, rheumatism and asthma and also as a diuretic, nerve tonic and cardiotonic in Ayurvedic medicine. It grows in marshy and damp areas and is a creeper plant with bitter taste. Saponins and triterpenoid bacosaponins that include bacopasides III to V, bacosides A and B, and bacosaponins A, B, and C are the main constituents of *Bacopa monnieri.* The jujube geninbisdesmosides bacopasaponins D, E, and F are the other saponin glycosides. Antioxidant activity is shown by the other components of this species, which include, alkaloids, plant sterols, betulic acid, polyphenols, and sulfhydryl compounds. This species has lipoxygenase inhibiting activity and can reduce divalent metal, scavenging reactive oxygen species and also decreases the formation of lipid peroxides. It also inhibits cholinergic degeneration

and displayed a cognition-enhancing effect in a rat model of AD. In improvement of cognitive function and memory it was used traditionally. For the nootropic actions and neuropharmacological effect it has been extensively investigated. Thus it is a good source of medicine in the treatment of AD.

d. *Centella asiatica* (Gotu Kola)

Gotu kola belonging to the Umbelliferae family has been used as an alternative medicine in Ayurvedic system for improving memory and increasing longevity and intelligence. It is an important herb used as rejuvenator for brain cells and nerve. It has a very important role in the treatment and prevention AD and beta amyloid toxicity as it has shown the capability of decreasing free radical concentrations and hydrogen peroxide-induced cell death. It also inhibits beta amyloid cell death in vitro.

e. *Gingko biloba*

For Alzheimer's disease and its associated symptoms *Ginkgo biloba* of the Ginkgoaceae family, native to China is best known. According to a clinical trial where a placebo and control group was used, it shows therapeutic benefits similar to prescription drug of Alzheimer's disease such as Donezepil or Tactrin. It showed therapeutic benefits with less undesirable side effects. *Gingko biloba* protects against Aβ protein-induced oxidative damages (degrading hydrogen peroxide, preventing lipids from oxidation, and trapping the reactive oxygen species). It also has the capability to systematically increase circulation. It has actions that is related to vasorelaxation and inhibit platelet aggregation along with lowering of blood pressure. Gingkolides is the main chemical constituent of *Gingko biloba* which is a pertinent antioxidant and has cholinergic and neuroprotective activities which is used in the management of AD. The extract of *Gingko biloba* also contains 24% flavonoids and 6% terpene lactones. In the early stages of AD its use has shown promising effect on cognition enhancement. *G. biloba* extract significantly inhibit the AChE activity in the brain. (Jivad, N. and Rabiei, Z., 2014)

f. *Rosmarinus officinalis* (Rosemary)

Rosmarinus officinalis also known Rosemary (*Satapatrika)*, can be used in the treatment of AD as a medicinal plant as it contains anti-inflammatory compounds and antioxidants of about nearly more than a dozen. It also contains several natural COX-

2 inhibitors, Apigenin, carvacrol, eugenol, oleanolic acid, thymol, and ursolic acid. Carnosic acid and ferulic acid are the two strong antioxidants present in the herb.

g. *Panax ginseng*

Ginseng, found in Northeastern Asia has many uses which helps in maintaining both physical and mental health. From ancient times, the root of Ginseng has been used in China and Korea in folk medicine. It has been reported, *Panax ginseng* has memory enhancing action for the learning impairment induced by scopolamine as it contains saponins, protopanaxadiol, protopantriol, and oleanolic acid saponins. According to research, Ginseng has the ability to increase cognitive and psychomotor performance. In the treatment of AD it can be used as it is capable of improving brain cholinergic function and repairing the damaged neuronal networks and thus reducing the level of AD.

h. *Bertholettia excelsa* (Brazil nuts)

Bertholettia excelsa is known as Brazil nuts which contains Lecithin in high concentration, which contains choline. Choline being the building block for acetyl-choline, has the ability to enhance the concentration of acetylcholine in AD patients and can be used in the treatment of AD.

i. *Magnolia officinalis*

For the treatment of neurosis, anxiety, stroke, and dementia in Chinese medicine, the bark of *Magnolia officinalis* has been used as a traditional medicine for enhancing memory. It has the capability to inhibit scopolamine induced memory impairment through AChE inhibition. Antioxidant activity has been shown by the ethanolic extracts of *Magnolia officinalis.* (Singhal, A.K., et. al, 2012)

j. *Lavandula officinalis* (Lavender)

Lavender belongs to the genus *Lavandula* which is known as lavender and used as essential oil in perfumes and cosmetics industry. It has pleasant smell with a bitter taste. There are several compounds detected in lavender extract, such as, geraniol, linalool, linalyl acetate, cineol, borneol, aflapin, camphor, butyric acid, valerianic acid, ursolic acid, and luteolin flavonoids, which may increase effects on central

nervous system area and causing calming and soothing effects through GABA receptor. Strong neuroprotective effect has been shown by treatment with lavender along with decrease in neurologic deficit. Different concentration of lavender extract has shown inhibitory effects on the AChE enzyme which have been proved through various tests on cell lines. Ethanol extract of lavender improves spatial learning and memory, motor coordination and passive avoidance learning. The neuroprotective effects may be due to its antioxidant properties.

k. *Salvia officinalis* (Sage)

Salvia officinalis includes anti-inflammatory and antioxidant properties as well as weak AChE inhibitory effect which is the potential pharmacological effects of the plant and relevant to AD. It has good effect on improving memory. For their antioxidative properties, the leaves of *S. officinalis* L. are used and very well known. A number of deleterious events induced by Aβ include reactive oxygen species formation, lipid peroxidation, DNA fragmentation, caspase-3 activation and tau protein hyperphosphorylation was reduced by the main active constituent of *Salvia officinalis,* Rosmarinic acid.It may help to prevent symptoms of AD, according to some clinical evidence. In a randomized double-blind clinical study, patients with mild-to-moderate AD received *S. officinalis* extract. The result showed that *S. officinalis* had statistically significant effectiveness in the cognition after 16 weeks of treatment. One small pilot trial showed that oral administration of *S. officinalis* essential oil to 11 patients showing mild-to moderate symptoms of AD significantly improved cognitive function. (Jivad, N. and Rabiei, Z., 2014)

2.1. Citrus Plant

Citrus, it is a genus of flowering trees and shrubs belonging to the Rutaceae family. The plants of this genus produce citrus fruits, important crops such as, lemons, oranges, limes and grapefruit. According to the most recent research, the origin is in Australia, New Guinea and New Caledonia. Some researchers indicate that the part of Southeast Asia bordered by Northeast India, Myanmar and the Yunnan province of China is the origin. Since ancient times citrus fruits has been cultivated in an ever-widening area.

2.2. History of Citrus fruits :

Citrus plants were thought to be native to Asia, where they were first domesticated, Europe, and Florida. But the 'native' oranges of Florida actually originated with the Spanish Conquistadors whereas the European oranges such as the bitter orange originated from India at around the time of Alexander the Great. During the time of classical Rome, the lemon reached Europe.

The generic name originally came from Latin, where it was referred to the plant which is now known as Citron (*Citrus medica*) or a conifer tree. Somehow it is related to the ancient Greek word for cedar. The reason may be because of the perceived similarities of the smell of citrus leaves and fruit with that of cedar. In the citrus genus, the mandarin orange, citron and pummelo are the three original species that have been hybridized into most modern commercial citrus fruits. By crossing these original species, all common citrus fruits, for example, lemons, sweet oranges, limes, grapefruits were created within the last few thousand years.

Citrus aurantifolia, Citrus grandis, *Citrus reticulate and Citrus limon* – these species are cultivated extensively for their taste, good pulp and have very high market demand. They have maximum diversity in South and western hills of Meghalaya in the North-East.
Citrus indica is known as Memang Narang locally in India and maybe the progenitor of cultivated Citrus. It is the most primitive species and also a rare one which is confined to the Tura ranges of West Garo Hills. (Uddin, N. et. al, 2014)

Citrus macroptera is reported to grow in the Khasi and Garo Hills of Meghalaya, North Cachar, Karimganj and Karbi-Anglong districts of Assam and the states of Mizoram, Tripura and Manipur.
Citrus megaloxycarpa is a rare species which is locally known as 'Sishupal' and confined to the Jampui Hill regions of Mizoram and *Citrus latipes* shows maximum occurrence in West Khasi Hills of Meghalaya.

The genus Citrus belonging to the family Rutaceae is economically very important which includes 162 species. Its is grown in tropical and subtropical areas of the world and is known for its pulp and juice all over the world. *Citrus* is the third most important fruit crop of India. Lemon, sweet orange, mandarin, acid lime are the major cultivated species of India. Other species which are cultivated to a lesser amount are pummelo, seedless lime, grapefruit and belladikithuli. There are 30 species of *Citrus* in India.

The regions that are important as the origin of *Citrus* and related genera are the south-east Asia, Australia and the intervening island-areas between Australasia and Central Africa and the north-eastern region of India along with neighboring China. Many *Citrus* species are thought to be endemic to the region. Seven Indian *Citrus* species fall under the category of endangered species which include *Citrus indica*, *Citrus macroptera*, *Citrus latipes*, *Citrus assamensis*, *Citrus ichangensis*, *Citrus megaloxycarpa* and *Citrus rugulosa*. Due to endemism and high degree of threat perception, *Citrus indica* and *Citrus macroptera*, these two species need immediate attention and special care. (Hynniewta, M., et. al, 2011)

2.3. *Citrus macroptera*

Citrus macroptera is a semi-wild species of citrus genus which is native to Malesia and Melanesia. Its other names are wild orange, Melanesian papeda, satkara or cabuyao. It was discovered on the Island of Art which is situated a few miles to the northwest of the north end of New Caledonia by Father Montrouzier. It was a tree growing near the houses of the natives and of about 15-16 feet high.

Citrus macroptera belongs to the family of Rutaceae. It is native to the regions of Southeast Asia mainly Myanmar, Thailand, Indonesia, Malaysia, Papua New Guinea, New Caledonia, Northeastern Bangladesh and northeastern India mainly Manipur and Assam.

In Bangladesh, it mainly grows in the Sylhet Division and locally known as 'hatkora' or 'shatkora'. The people of Bangladesh eat its fruit as a vegetable. The fruit is edible and is used as ingredient in cooking different kinds of meat and chicken. It is popular among the people of Bangladesh, Meghalaya and Assam of India as green matured fruits are used for flavoring in cooking curry, meat dishes and pickle preparation. Traditionally, the fruit is used as appetite stimulant and in treatment of fever. (Uddin, N. et. al, 2014)

2.4. *Citrus macroptera* Tree

The tree of *Citrus macroptera* is perennial and woody plant with a single stem or trunk. It has thorns and can be greater than 13 to 16 feet (4-5 meters) in height. Some tree species may develop a multi stemmed or short growth form under certain environmental conditions.

Citrus macroptera has large leaves having the width of 2 inches and sometimes 10 or 12 inches long. It has very large winged petiole leaves for which this species is so named as *Citrus macroptera.* (Plants.usda.gov., 2016)

Figure 2.1 : *Citrus macroptera* Plant

2.5. *Citrus macroptera* Fruit

The fruit of *Citrus macroptera* is about 6–7 cm in diameter. It has a fairly smooth and moderately thick rind, and becomes yellow when ripen. The pulp is greenish yellow and dry, having little juice which is very sour, and bit bitter. (Rahman, H., et. al, 2014)

Figure 2.2 : *Citrus macroptera* fruit

2.6. The Scientific Classification of *Citrus macroptera*

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Rosidae
Order	Sapindales
Family	Rutaceae
Genus	*Citrus*
Species	*Citrus macroptera*

(Plants.usda.gov., 2016)

Table 2.1 : Scientific Classification of *Citrus macroptera*

2.7. Literature Review

1. Waikedre J et. al says, the *Citrus macroptera* leaf oil exhibited a pronounced activity against Trichophyton mentagrophytes var. interdigitale, with a minimal-inhibitory concentration (MIC) of 12.5 microg/ml. The essential oil of *Citrus hystrix* was characterized by high contents of terpinen-4-ol (13.0%), alpha-terpineol (7.6%), 1,8-cineole (6.4%), and citronellol (6.0%). The antimicrobial activity was evaluated against five bacteria and five fungi strains. Both oils were inactive against bacteria. As there is a relation between these plants, that is why we were interested for this work. (Waikedre, J, et. al, 2010)

2. Rana VS, Blazquez MA says, the chemical compositions of the essential oils obtained by hydrodistillation from the fresh peels of *C. macroptera Montr.* and *C. maxima* were found to be similar, but nootkatone (1.6-2.5%) was identified only in *C. maxima* cultivars. This is related to the tests and the plant, thus we were influenced to work on this. (Rana, V.S. and Blazquez, M.A., 2012)

3. Dang, N.H., et. al says, among the citrus essential oils, the Buddha's hand oil showed the most significant α-glucosidase inhibitory effect with the IC_{50} value of 412.2 μg/mL. The combination of the Buddha's hand essential oil and the antidiabetic drug acarbose increased the inhibitory effect. Conclusions. The results suggested the potential use of Buddha's hand essential oil as an alternative in treatment of type 2 diabetes mellitus. As in this test citrus oil is used which is related to our fruit/plant, thus we were interested for this work. (Dang, N.H., et. al, 2016)

4. Ahmad I et. al says, both titrable acidity and vitamin C content of *C. macroptera* and *C. assamensis* are sensitive to both gamma radiation and storage time and, have decreased with increase of radiation as well as storage time and this changes of vitamin C and titrable acidity content with gamma radiation and increasing storage period have found statistically significant. (Ahmad, I., et. al, 2013)

5. Gaillard, E. et. al says, a preliminary screening showed the occurrence of alkaloids only in stem bark of *C. macroptera Montr*. In a first work on this Citrus, only one alkaloid, edulinine was identified. The isolation of two other alkaloids: (+/-) Ribalinine and Isoplatydesmine and of five aromatic compounds: two derived from cinnamic acid, three identified as syringaldehyde, vanillin and methyl/vanillinate were reported. (Gaillard, E., et. al, 1994)

6. Uddin N et. al says, Presence of saponin, steroid and terpenoid were identified in the extract of *C. macroptera* fruit. The results showed that fruit extract had moderate α-amylase inhibitory activity [IC50 value =3.638±0.190 mg/mL] as compared to acarbose. (Uddin, N., et. al, 2014)

7. Miah M.N et. al says, Somatic embryogenesis and plantlet regeneration were achieved in callus cultures of nucellus derived from undeveloped ovule of immature fruits of *C. macroptera.*(Miah, M.N., et. al, 2002)

8. Malik, S.K. and Chaudhury, R. says, successful cryopreservation of embryonic axes of two wild and endangered species, *Citrusmacroptera* Mont. and *C. latipes* Tanaka, was achieved using air desiccation–freezing, vitrification and encapsulation–dehydration. *C. macroptera* was more tolerant to desiccation and freezing than *C. latipes* with recovery rates of, respectively, 87% and 64%. The air desiccation–freezing protocol being a simple and practical technique is recommended for the cryopreservation of these two species. (Malik, S.K. and Chaudhury, R., 2006)

9. Rahman, H., et. al says, Ethanolicextract of *C. macroptera* fruit peels (EECM) found to possesanti-depressant and anxiolytic but does not possess anti-epileptic rather it found to potentiate epileptic Seizurein mice.(Rahman, H., et. al, 2014)

10. Marak, C.K. and Laskar, M.A., says, Principal Components Analysis

projected *C. indica* to be more closely related to *C. aurantifolia* than to the other citrus species of the study. PCA also exposed variations within the *C. maxima* and *C. macroptera* samples. (Marak, C.K. and Laskar, M.A., 2010)

11. Panara, K says, the test result shows that *Citrus medica* Linn. possesses analgesic, hypoglycaemic, anticholinesterase, anticancer, antidiabetic, hypocholesterolemic, hypolipidemic, insulin Secretagogue, anthelmintic, antimicrobial antiulcer and estrogenic properties. (Panara, K., 2012)

12. Lota. M.L., et. al says, Three chemotypes: limonene, limonene/γ-terpinene and limonene/geranial/neral were observed for five peel oils while six leaf oils of different varieties of citrons (*Citrus medica* and *C. limon*) exhibited the limonene/geranial/neral composition. (Lota, M.L., et. al, 1999)

13. Sood, S., et. al says, Ethyl acetate extract of *Citrus medica* peel (EtCM) showed maximum 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydrogen peroxide radical scavenging activity in a dose dependent manner as compared to ascorbic acid. *Citrus medica* peel extract may be used as a future antioxidant for the treatment of inflammation and pain.(Sood, S., et. al, 2009)

14. Menichini, F., et. al says, the antioxidant activity of *C. medica* evaluated by the β-carotene bleaching test showed a strong activity for flowers and endocarp of mature fruits with IC_{50} values of 2.8 μg/mL and 3.5 μg/mL, respectively, after 30 min of incubation. Interestingly, the mature fruits endocarp (IC_{50} value of 426.0 μg/mL) could inhibit α-amylase with an IC_{50}value 2-fold higher than immature fruits. None of the tested extracts affected the proliferation of human skin fibroblasts 142BR. The obtained results suggest a potential use of *C. medica* L. cv Diamante as new valuable Citrus species with functional properties for food or nutraceutical product on the basis of high content of phytochemicals. (Menichini, F., et. al, 2011)

15. M Viuda-Martos et. al says, The objective of this work was to study the effect of the essential oils of lemon (*Citrus lemon* L.), mandarin (*Citrus*

reticulata L.), grapefruit (*Citrus paradisi* L.) and orange (*Citrus sinensis* L.) on the growth of moulds, using the agar dilution method. All the essential oils of lemon (*Citrus lemon* L.), mandarin (*Citrus reticulata* L.), grapefruit (*Citrus paradisi* L.) and orange (*Citrus sinensis* L.) showed antifungal activity against all the moulds commonly associated with food spoilage: *Aspergillus niger*, *Aspergillus flavus, Penicillium chrysogenum* and *Penicillium verrucosum.* Orange essential oil was the most effective against *A. niger*, mandarin essential oil was most effective at reducing the growth of *Aspergillus flavus* while grapefruit was the best inhibitor of the moulds *P. chrysogenum* and *P. verrucosum.* Citrus essential oils could be considered suitable alternatives to chemical additives for use in the food industry. (Viuda-Martos, M., et. al, 2008)

16. Chutia, M., et. al says, the essential oil (EO) isolated by hydro-distillation from the peel of fully matured ripen fruits of *Citrus reticulata* Blanco showed better activity in VA assay. Fungal sporulation was also completely inhibited at 2 ml/100 ml of the oil except for *Curvularia lunata* and *Helminthosporium oryzae*. (Chutia, M., et. al, 2009)

17. Lota, M.L., et. al says, Peel and leaf oils of 41 mandarin cultivars, belonging to the *Citrus reticulata* Blanco species, were obtained from fruits and leaves collected on mandarin trees submitted to the same pedoclimatic and cultural conditions. Two major chemotypes, limonene and limonene/γ-terpinene, were distinguished for peel oils while three major chemotypes, sabinene/linalool, linalool/γ-terpinene and methyl N-methylanthranilate, were observed for leaf oils. (Lota, M.L., et. al, 2000)

18. L.M Lopes Campêlo et. al says, sedative and anxiolytic effects of EO of leaves of Citrus limon might involve an action on benzodiazepine-type receptors, and also an antidepressant effect where noradrenergic and serotoninergic mechanisms will probably play a role. (Lopes Campêlo, et. al, 2011)

19. Rekha, C., et. al says, unripe fruit juices of *Citrus limon*, *C. reticulata*, *C. sinensis* and *C. aurantium* have displayed stronger antioxidant activity when compared to ripe fruit juices. The antioxidant activity of fruit juices was shown to be directly related to the content of ascorbic acid and total phenolics except in case of *C. aurantium*. The lower antioxidant activity of ripe fruit juices could be due to the possible reduction in the ascorbic acid and total phenolic content during ripening. (Rekha, C., et. al, 2012)

20. S Kaviya et. al says, Biosynthesis of silver nanoparticles (AgNPs) was achieved by a novel, simple green chemistry procedure using citrus sinensis peel extract as a reducing and a capping agent. The results suggest that the synthesized AgNPs act as an effective antibacterial agent. (Kaviya, S., et. al, 2011)

21. P Singh et. al says, Essential oils (EOs) of *Citrus maxima* and *Citrus sinensis* exhibited antioxidant activity as DPPH free radical scavenger in dose dependent manner. The oils may be recommended as safe plant based antimicrobials as well as antioxidants for enhancement of shelf life of food commodities by checking their fungal infestation, aflatoxin production as well as lipid peroxidation. (Singh, P., et. al, 2010)

22. 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, hepatoprotective, antioxidative property, cytotoxic activity, and many more medicinal values. For these activities we are interested to work on *Citrus Maxima.* (P. Vijaylakshmi and R. Radha, 2014)

23. RA Onyeagba et al says *Citrus aurantifolia* has the antimicrobial activity against *Staphylococcus aureus*; *Bacillus spp., Escherichia coli* and *Salmonella spp.* This creates interest for doing this work.

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

25. J.R Patil et. al says, Limonexic acid, β-sitosterol glucoside and limonin glucoside isolated from *Citrus aurantifolia* (lime) seeds possess the mechanism of inhibition of human pancreatic cancer cells. (Patil, J.R et. al, 2010)

26. Loizzo, M.R., et. al says there is a potential use of *C. aurantifolia* peel and leaves for supplements for human health. (Loizzo, M.R., et. al, 2012)

27. Paul, S., et. al says, *C. macroptera* fruit could protect against acetaminophen-induced hepato nephrotoxicity, which might be via the inhibition of lipid peroxidation. (Paul, S., et. al, 2016)

28. Paul, S., et. al says, *C. macroptera* possesses high antioxidant potential and is relatively safe. Its protective effects against various chronic diseases that are associated with oxidative stress should be further investigated. (Paul, S., et. al, 2015)

3.1. Collection of Plant Materials

The fruits of the *Citrus macroptera* were collected from Sylhet Division which is a district of Bangladesh, in March, 2016.

3.2. Preparation of Plant Materials

Initially, the fruits were washed with water to eradicate sticky dirt and was cut into pieces. The peel and pulp were separated and cut into pieces. Then the peel and pulp were subjected to shade dry for numerous days with random sun drying. These samples were then dried in an oven for a day at significantly low temperature in order to better grinding. The dried peel and pulp were crushed into granular powder by a crushing machine.

Fig 3.1: *Citrus macroptera* powder (Pulp)

Fig 3.2 : Dried pulp of *Citrus macroptera*

Fig 3.3 : *Citrus macroptera* powder (Peel)

Fig 3.4 : Dried Peel of *Citrus macroptera*

3.3 Extraction of the Plant Materials

Grinded plant materials (peel and pulp) were taken in an amber colored reagent bottle. Weight of powdered peel was 500gm and weight of powdered pulp was 250gm. The total amount of peel was soaked in 3.0 liter of methanol and the total amount of pulp was soaked in 2 liter of methanol.

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

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

Name of the fractions	Weight of the fractions (gm)
Peel (CME)	50.5gm
Pulp (CME)	42.5gm

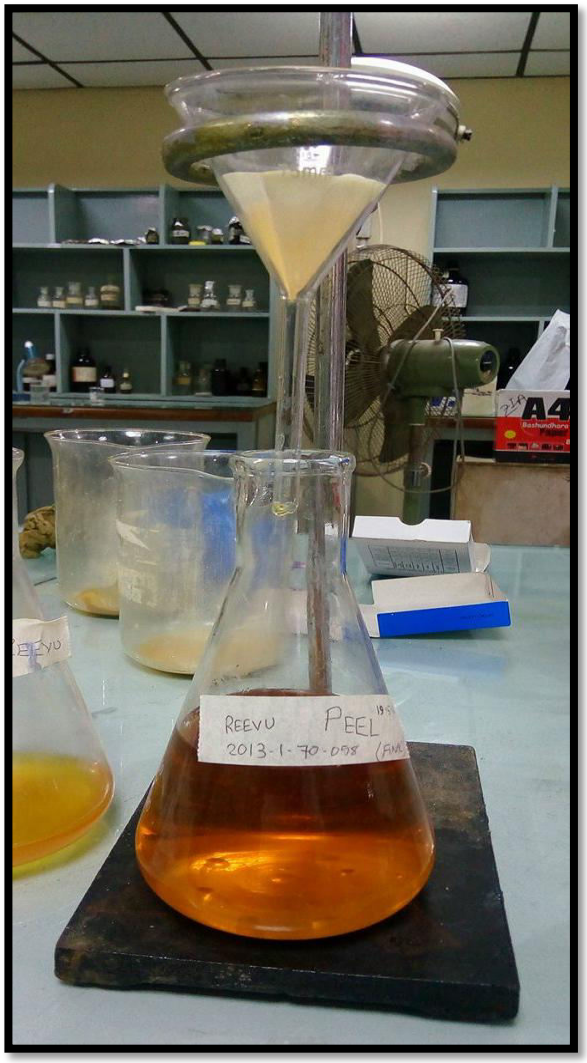

Figure 3.5: Filtration of *Citrus macroptera* extract (Peel)

Figure 3.6 : Filtrate of *Citrus macroptera* Pulp (on left) and Peel (on right)

3.4 Total Phenolic Content Determination

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

Principle

The amount of total phenolic compounds of various fractions in the plant was identified by Folin–Ciocalteu Reagent (FCR). The FCR is the parameter of measuring reducing capacity of a sample. The accurate chemical nature of the FC reagent is unknown, but it is assumed to contain hetero polyphosphotungstates–molybdates. Sequences of not irreversible one or two-electron reduction reactions turns into to

blue species, probably (PMoW11O40)4. It is assumed that the molybdenum is at ease to be reduced in the complex and electron-transfer reaction takes place between reductants and Mo (VI).

Materials

- ✓ Folin – ciocalteu reagent (Sigma chemical company, USA),
- ✓ Sodium carbonate (Sigma chemical company, USA),
- ✓ Methanol (Sigma chemical company, USA),
- ✓ Gallic acid (Wako pure chemicals Ltd., Japan),
- ✓ Micropipette (100-1000 µl),
- ✓ Pipette (1-10 ml),
- ✓ UV-spectrophotometer.

Procedure

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

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

Where,

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

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

V = The volume of extract, ml;

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

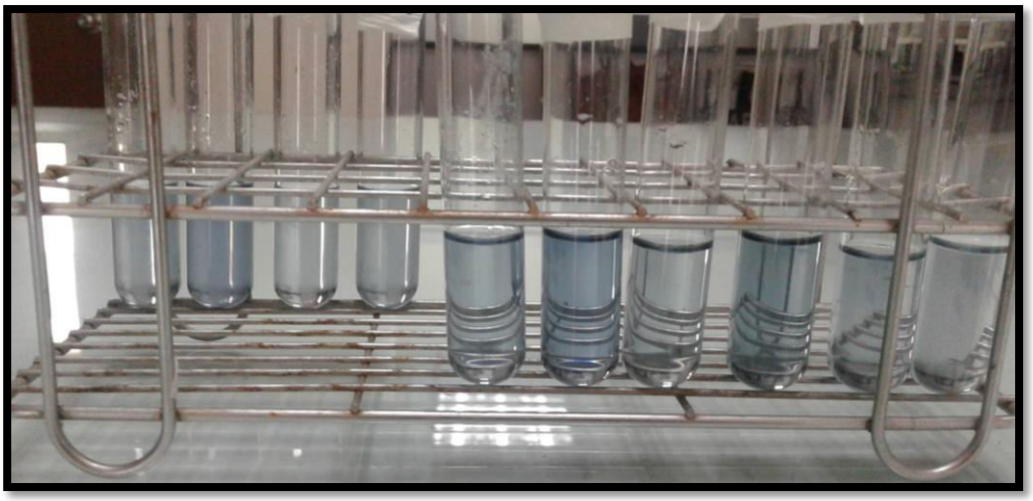

Figure 3.7 : Total Phenolic Content Determination Test

3.5 Determination of Total Flavonoids Content

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

Principle

The amount of total flavonoid content in various extracts of plant was identified by the popular aluminum chloride colorimetric method. In this method, aluminum chloride makes complex with hydroxyl groups of flavonoids which may be present in the samples. This complex has the maximum absorbance at 510 nm.

Materials

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

Procedure

Total flavonoid (TF) content was identified by using the procedure by Dewanto, Wu, Adom, and Liu. One milliliter of extract which contains 0.1 g/mL of dry matter was taken in a 10 mL volumetric flask. Then 2.5 mL of distilled water added and after that 0.15mL of 5% $NaNO_2$ was added. After 5 minutes, 0.3 mL of 10% $AlCl_3$ was added. Then after another 5 minutes, 1 mL of 1M NaOH was added with distilled water. The solution was mixed and absorbance taken at 510 nm. TF amounts were stated as catechin equivalents per dry matter.

The total amount of flavonoid compounds in plant extracts in catechin equivalents was calculated by the following formula equation

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

Where,

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

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

V = The volume of extract, ml;

m = The weight of pure plant extracts, gm.

Figure 3.8 : Total Flavonoids Content Determination Test

3.6 Total Flavanol Content Determination

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

Principle

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

Materials

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

Procedure

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

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

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

Where,

C = Total content of flavonoid compounds, mg/g plant extract, in catechin equivalent (GAE);
c = The concentration of catechin established from the calibration curve, mg/ml;
V = The volume of extract, ml;
m = The weight of pure plant extracts, gm.

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

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

Principle

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been extensively used to assess the free radical scavenging capability of antioxidants. DPPH free radical is reduced to the hydrazine when reaction occurs with hydrogen donors. DPPH is able to make stable free radicals in solution of methanol or water. By this method it was possible to identify the antiradical power of an antioxidant activity by measuring the reduction in the absorbance of DPPH which was taken at 517 nm. This results in a change of color from purple to yellow. The absorbance reduced when the DPPH was scavenged by an antioxidant, through the donation of hydrogen to form a stable DPPH molecule. In the radical form this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.

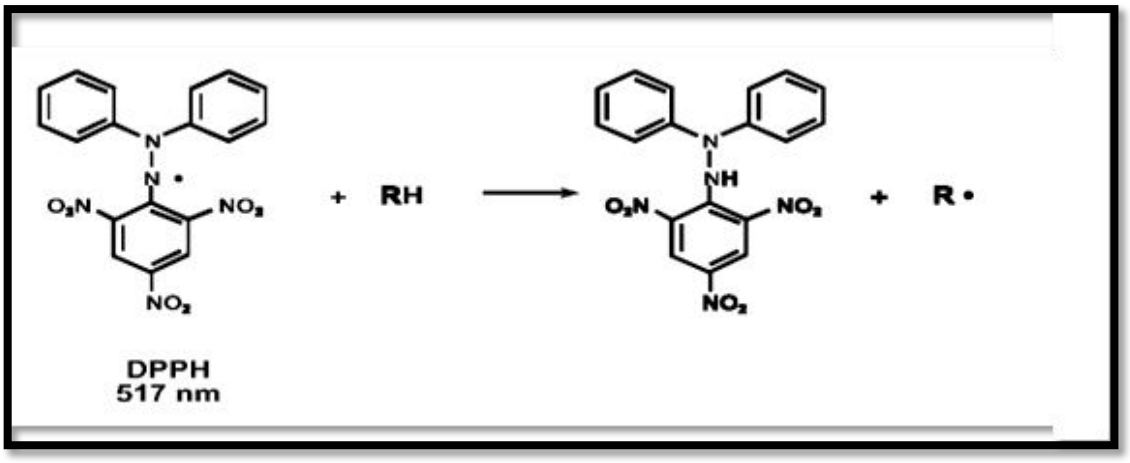

Figure 3.9: Mechanism of DPPH˙ with an antioxidant having transferable hydrogen radical.

Materials

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

Procedure

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

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

Where,

I% is the percentage of scavenging activity

A0 is the absorbance of the control, and

A1 is the absorbance of the extract/standard.

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

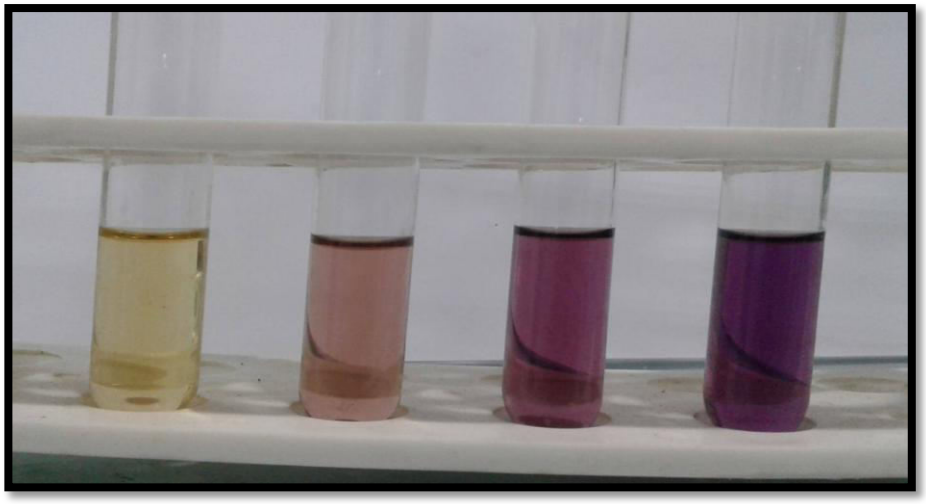

Figure 3.10 : Test DPPH Free Radical Scavenging Activity

3.8 Assessment of Reducing Power Capacity

The reducing power of the different extractives from extracts of *C. macroptera* evaluated by the method of Oyaizu.

Principle

In this method, the yellow colored test solution changes to numerous shades of green and blue which depends on the reducing power of antioxidant samples. The reducing capacity of a compound might serve as an important indicator of its potential antioxidant activity. The presence of reductants such as antioxidant constituents in the samples is the reason behind the reduction of the Fe3+-ferricyanide complex to the ferrous form by giving an electron. The amount of Fe2+ complex can then be observed by measuring the formation of Perl's Prussian blue at 700 nm.

Fe3+-ferricyanide + e-Fe2+-ferricyanide

Materials

- ✓ Potassium ferricyanide (Merck, Germany)

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

Procedure

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

3.9 In-Vitro Acetyl Cholinesterase Inhibitory Studies

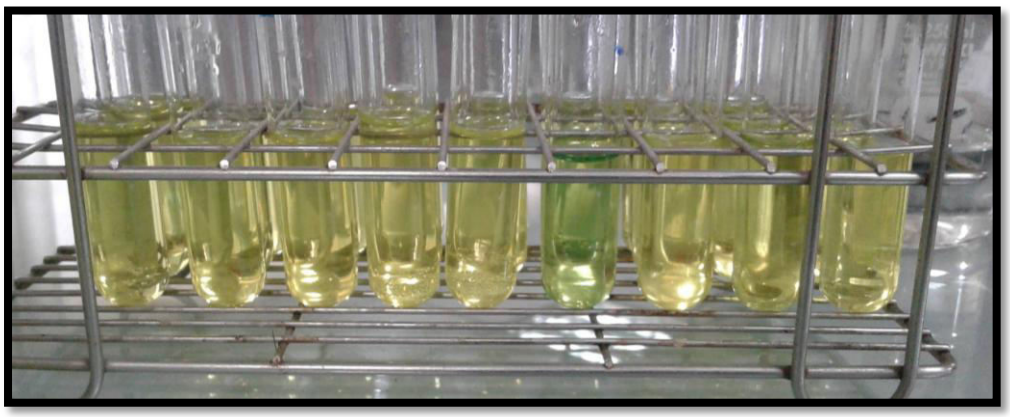

Figure 3.11 : Test of Assessment of Reducing Power Capacity

3.9.1 Purification of Acetyl Cholinesterase enzyme

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

Materials

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

3.9.2 Formulation of reagents

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

Procedure

3.9.3. Preparation of Crude enzyme extract

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

3.9.4. Precipitation with Ammonium Sulphate

The crude extract was precipitated by super saturated ammonium sulphate salt. Because of having low density, as compare to ammonium sulphate solution, the precipitate created to the surface on standing. Centrifuge this mixture at 3000 rpm for 25 min. The bottom layer was collected. At the terminal stage, the precipitate which was dissolved in homogenization buffer, used as a one-step purified source of enzyme.

Principle

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

Acetylcholine Thiocholine + Acetate = Thiocholine + dithiobisnitro Benzoate yellow color

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

Materials

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

Procedure

For positive control, different concentrations such as 20, 50, 100, 200 µl was added with 200 µl of enzyme. The solution was made upto 3ml with extraction buffer. This mixture was incubated for 20 minutes. Then 200µl of DTNB and 400µl of BTCI

solution was added. This was allowed to stand for 30 minutes at 37C. Absorbance was taken at 412 nm. For negative control, extraction buffer was added instead of enzyme.

3.10. In- Vitro Butyryl cholinesterase Inhibitory Studies

Principle

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

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

Materials

- ✓ 5, 5´-dithio-bis-(2-nitro) benzoic acid (DTNB) (Sigma-Aldrich, Japan),
- ✓ Butyrylthiocholine iodide (Sigma-Aldrich, Japan),
- ✓ Human blood plasma (Crude enzyme),
- ✓ Tris-Hcl buffer (Merck, Germany),
- ✓ Triton X-100 (Sigma chemical company, USA),
- ✓ Micropipette (100-1000 µl),
- ✓ UV spectrophotometer (Shimadzu, Japan),

Procedure

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

(0.7mM) and 400 µl BTCI (0.35mM) added correspondingly. Heat this for 40 minutes at 37C. For measuring the background BTCI was avoided. Reading was taken at 412nm. From the difference between BTCI positive and negative data the activity of extract was measured. The blank reaction was measured by substituting saline for the enzyme.

Figure 3.12 : Preparation of Butyryl cholinesterase enzyme

3.1 Test for Thrombolytic Activity

Thrombolytic activity of the methanoic extracts of peel and pulp of *C. macroptera* was estimated by using blood of human.

Principle

Thrombosis is referred as the clotting of blood in cardiovascular system. Clotting of blood in brain can play a significant role in the progression of Alzheimer's disease.

The thrombolytic activity of methanolic extract of *C. macroptera* was calculated by the given formula

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

Materials

- ✓ Human blood
- ✓ Eppendorf tube

Procedure

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

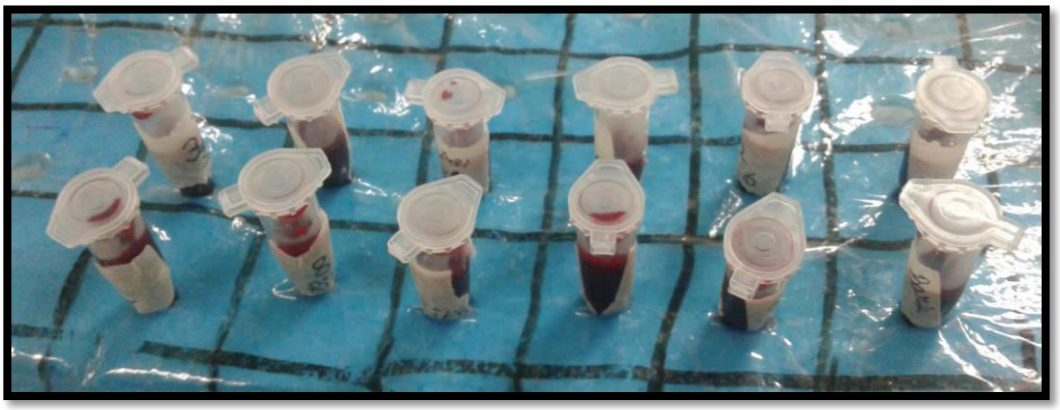

Figure 3.13 : Test for Thrombolytic Activity

4.1. Chemical Works

4.1.1. Preparation of Crude Methanolic Extract:

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

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

Name of the fractions	Weight of the fractions (gm)
Peel	20
Pulp	15

4.2. Determination of Total Phenolics:

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

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

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

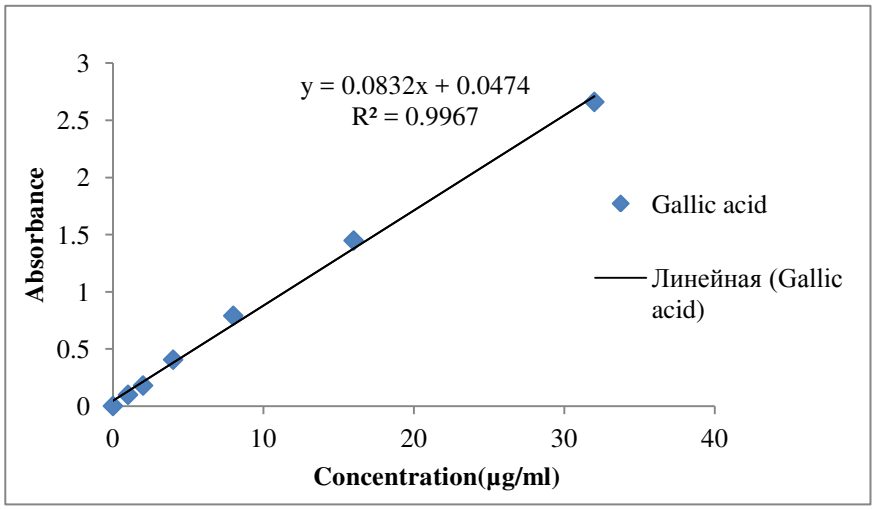

Figure 4.1 : Standard curve of Gallic acid for the determination of total Phenolics

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

Plant Name	Sample	Conc. (µg/ml)	Absorbance	GAE/gm of dried sample
Peel	**CME**	300	0.244	14.64
Pulp	**CME**	300	0.345	20.70

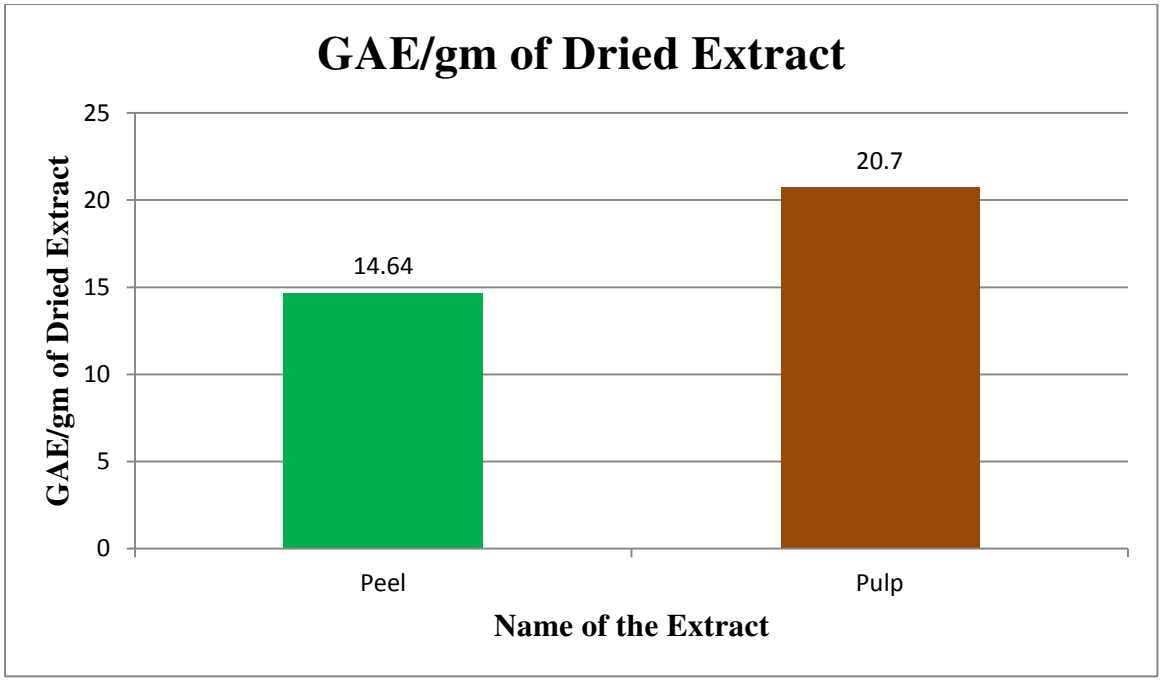

Figure 4.2 : Total phenol content (mg/gm plant extract in Gallic acid equivalent) of crude methanol extract

4.3. Determination of total Flavonoids of crude methanol extract (CME):

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

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

Concentration (µg/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

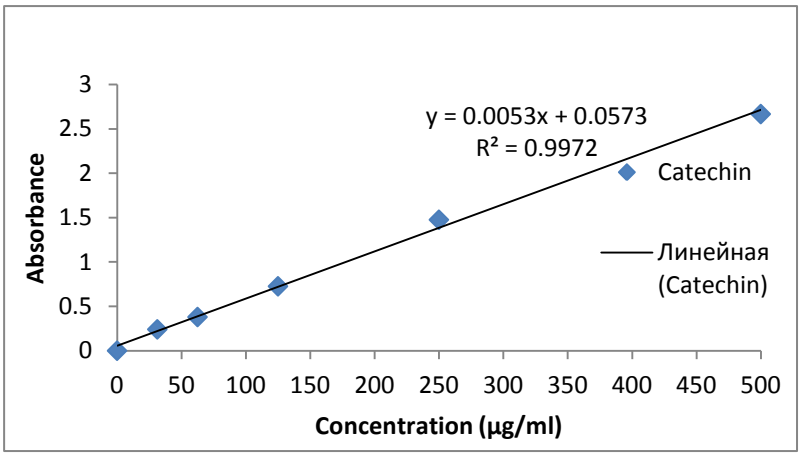

Fig. 4.3: Standard curve of Catechin for the determination of total flavonoids.

Table 4.5: Determination of total Flavonoid content of the crude methanol extract (CME)

Plant Name	Sample	Conc. (µg/ml)	Absorbance	CE/gm of dried sample
Peel	**CME**	300	0.024	1.44
Pulp	**CME**	300	0.029	1.74

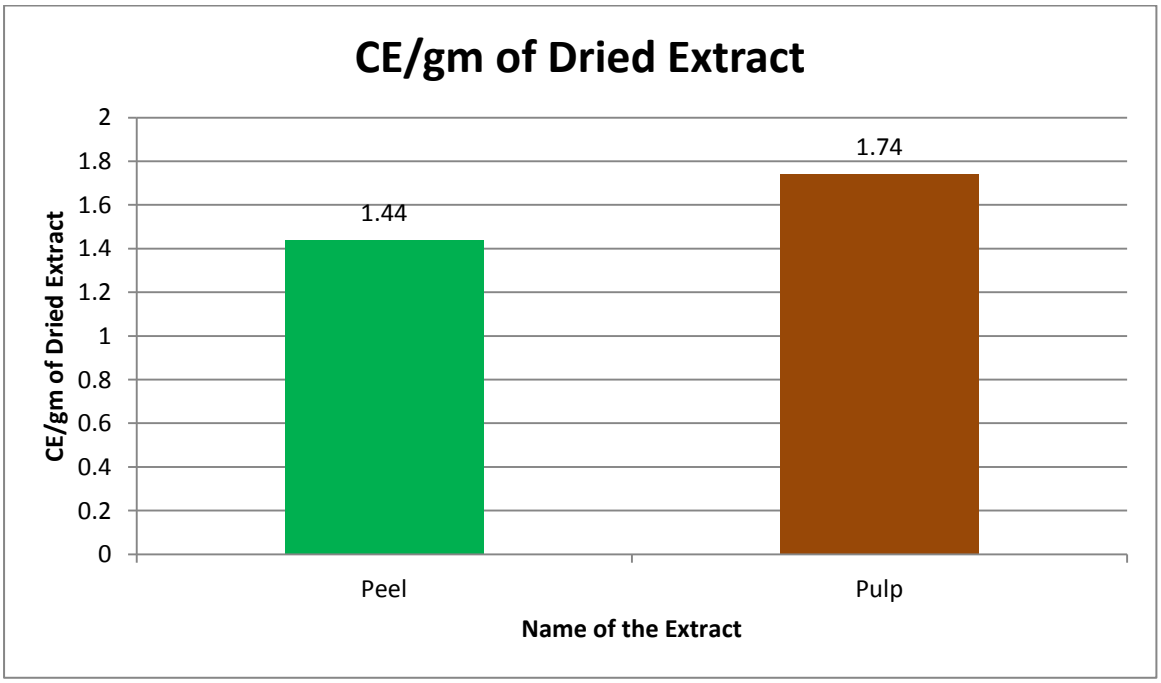

Fig 4.4: Total flavonoid content (mg/gm plant extract in Catechin equivalent) of crude methanol extract (CME)

4.4. Determination of total Flavanol

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

Table 4.6 :Absorbance of Gallic acid at different concentrations

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

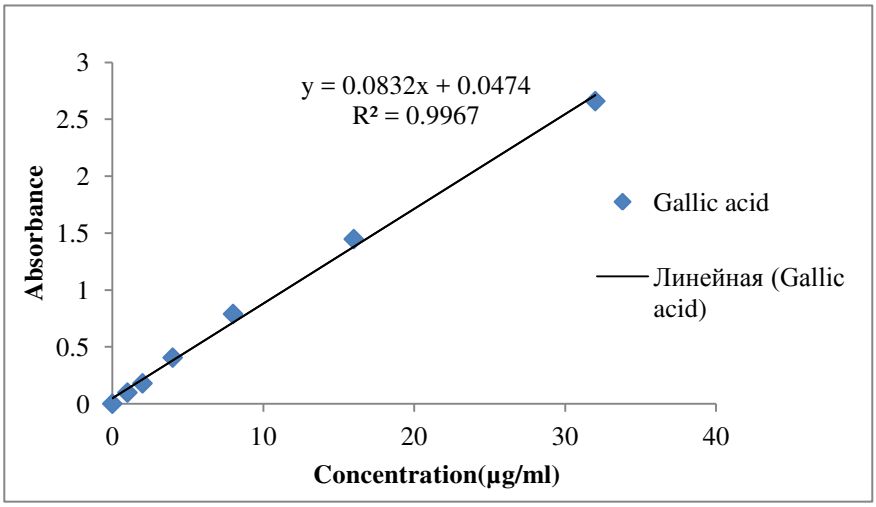

Fig 4.5 : Standard curve of Gallic acid for the determination of total Flavanols.

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

Plant Name	Sample	Conc. (µg/ml)	Absorbance	GAE/gm of dried sample
Peel	**CME**	300	0.098	2.52
Pulp	**CME**	300	0.039	1.14

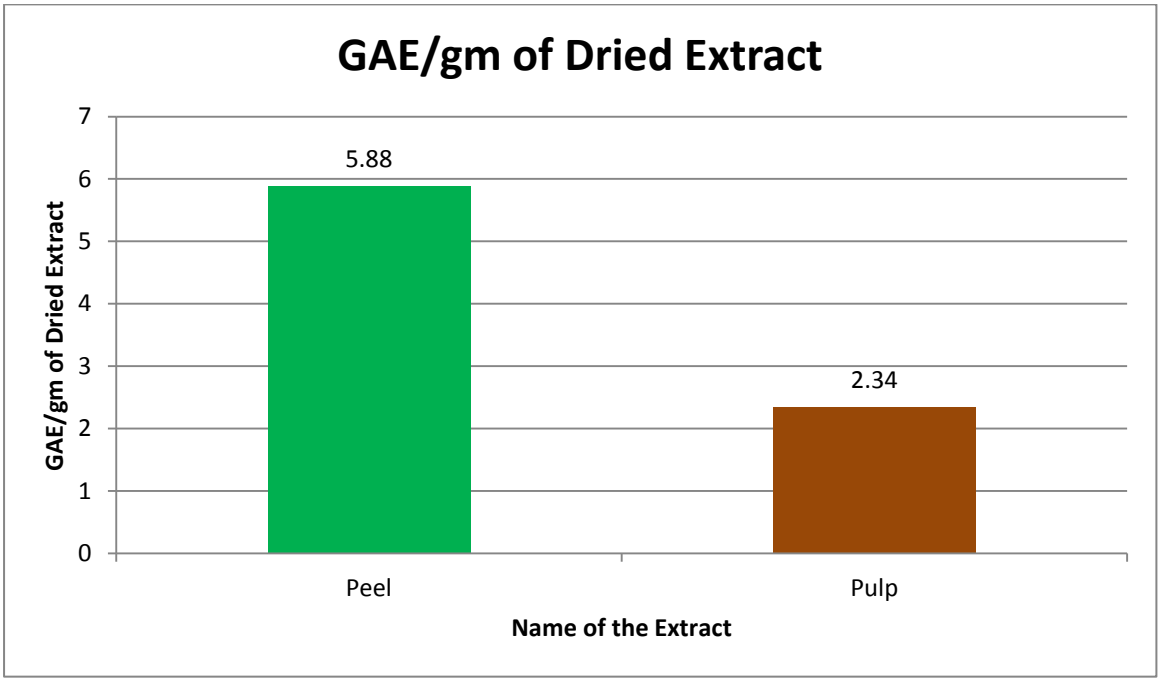

Fig 4.6: Total Flavanol content (mg/gm plant extract in gallic acid equivalent) of crude methanol extract

4.5. DPPH Radical Scavenging Activity

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

Table 4.8 : Determination of DPPH activity (CME)

Name of Plant	Concentration (µg/µg)	Absorbance	% of Inhibition
Peel	20	1.056	12.00
	50	1.018	15.17
	100	0.991	17.42
	200	0.980	18.33
Pulp	20	1.105	7.92
	50	1.073	10.58
	100	1.014	15.50
	200	0.989	17.58
Catechin (Standard)	20	0.19	87.39
	50	0.14	93.87
	100	0.11	94.16
	200	0.09	95.77

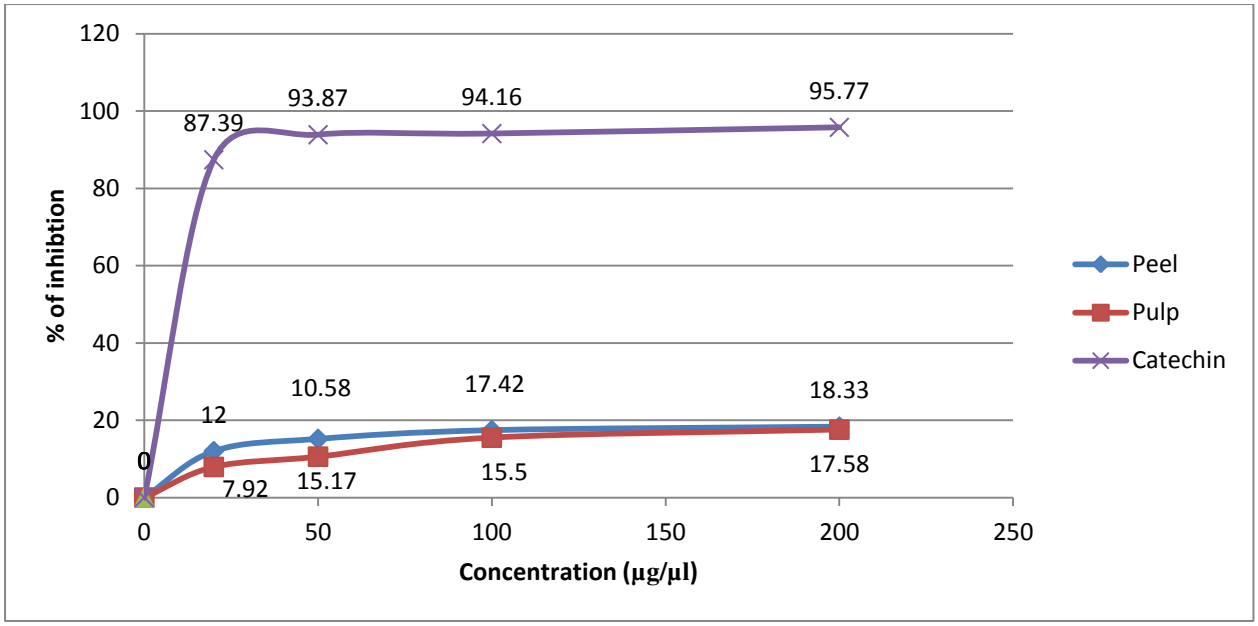

Fig 4.7: Determination of DPPH activity (CME)

4.6. Reducing Power Capacity:

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

Table 4.9: Determination of Reducing power capacity (CME)

Name of Plant	Concentration (µg/µg)	Absorbance
Peel	100	0.082
Pulp	100	0.8
Catechin (Standard)	100	2.660

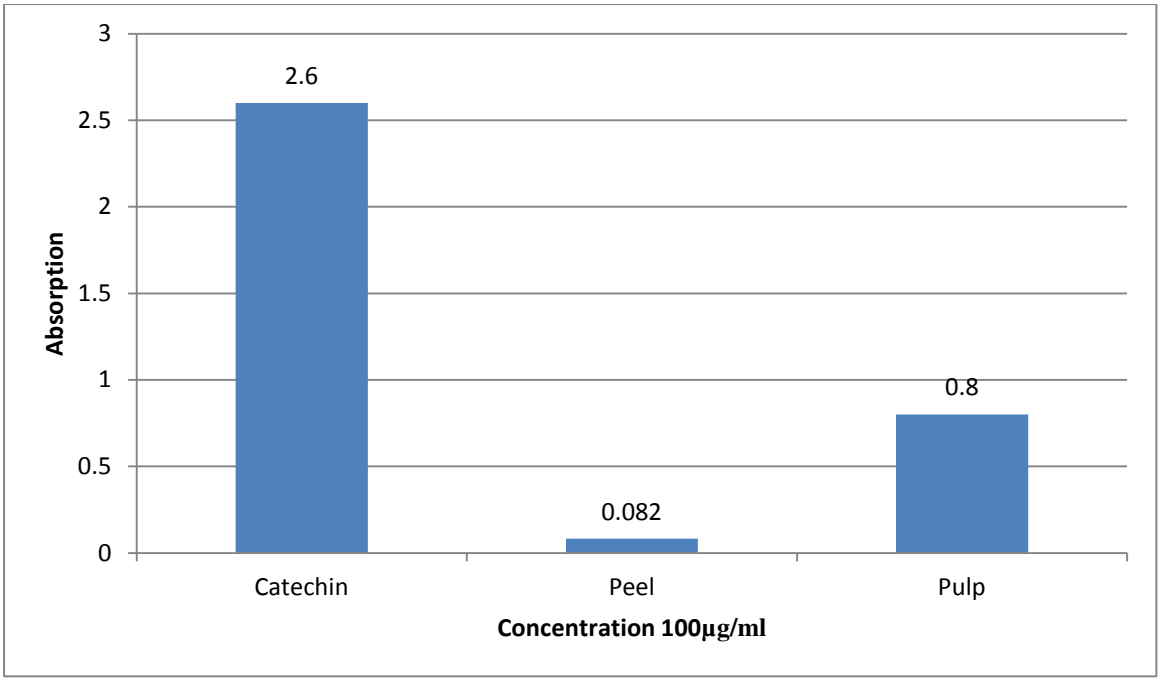

Fig 4.8: Determination of Reducing power capacity (CME)

4.7. Acetyl Cholinesterase Inhibitory Activity Assay

Inhibition of Acetyl Cholinesterase, 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 Acetyl Cholinesterase 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 Acetyl Cholinesterase (AChE) using acetylcholine iodide (substrate) and Dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with dithiobisnitro benzoate ion.

Table 4.10: Determination of Acetyl Cholinesterase inhibitory activity assay (CME)

Name of sample	Conc. (µg/ml)	% of inhibition Mean
Donepezil (Std)	20	78.34
	50	86.12
	100	91.44
	200	92.14
Peel	20	27.37
	50	34.87
	100	49.88
	200	75.63
Pulp	20	21.63
	50	35.00
	100	41.63
	200	69.25

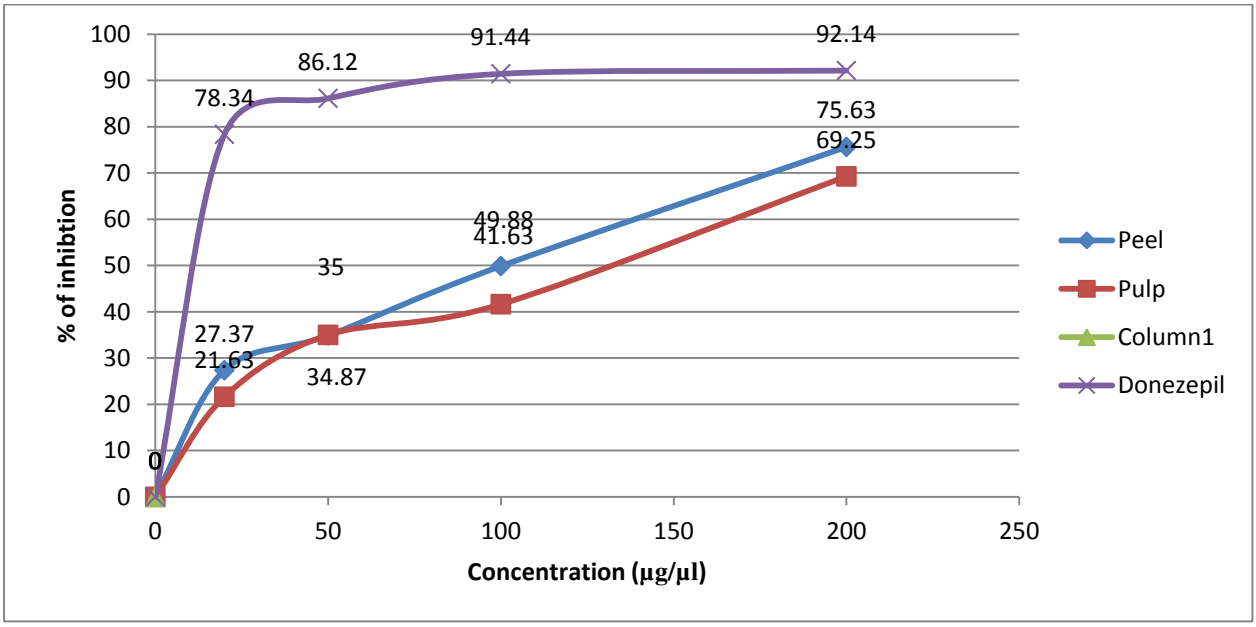

Fig 4.9 : Determination of Acetyl Cholinesterase inhibitory activity assay (CME)

4.8. Butyryl Cholinesterase Inhibitory Activity of Enzymes:

Butyryl Cholinesterase enhances cholinergic transmission by reducing the enzymatic degradation of both acetylcholine and butyrylcholine. Thus inhibition of Butyryl Cholinesterase 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. Butyryl Cholinesterase inhibitors have synergistic activity of Acetyl Cholinesterase inhibitory activity.

Table 4.11: Determination of Butyryl Cholinesterase inhibitory activity assay (CME)

Name of sample	Conc. (µg/ml)	% of inhibition Mean
Galantamine (Std)	20	72.43
	50	86.17
	100	88.42
	200	91.41
Peel	20	6.43
	50	10.00
	100	12.86
	200	16.43
Pulp	20	8.43
	50	17.50
	100	18.72
	200	46.57

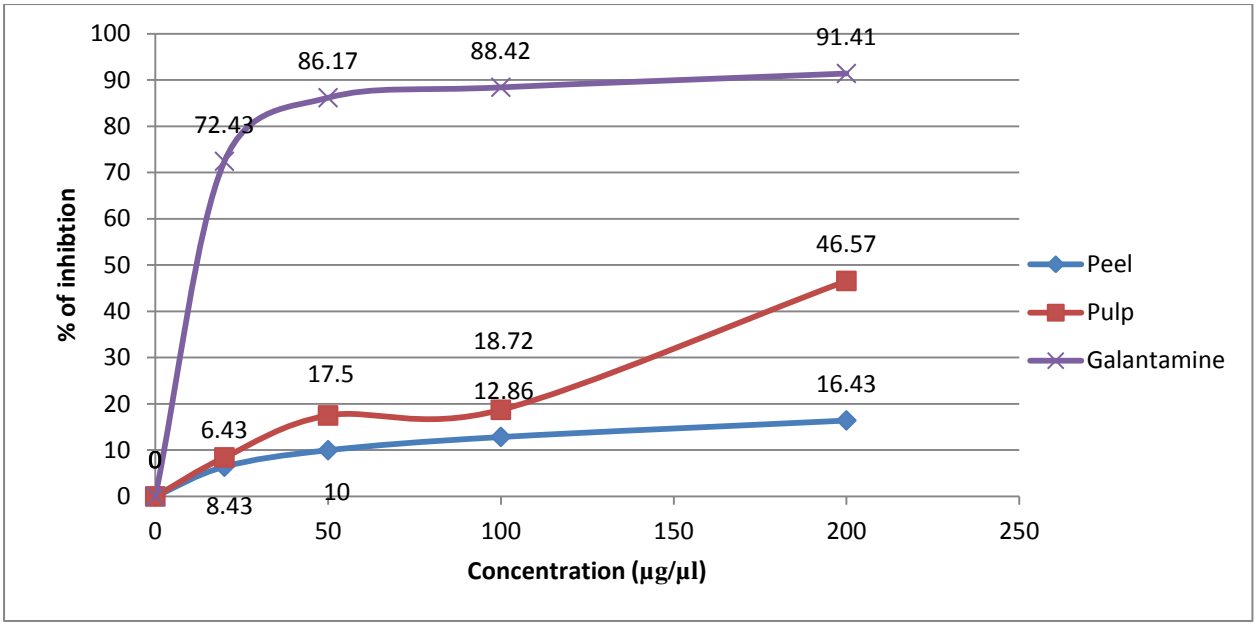

Fig 4.10: Determination of Butyryl Cholinesterase inhibitory activity assay (CME)

4.9. Thrombolytic Activity Test:

Table 4.12 : Determination of Thrombolytic Activity (CME)

Plant name	Concentration (µg/µg)	% of clot lysis
Peel	100	14.98
Pulp	100	4.76
Streptokinase	100	*87.01*

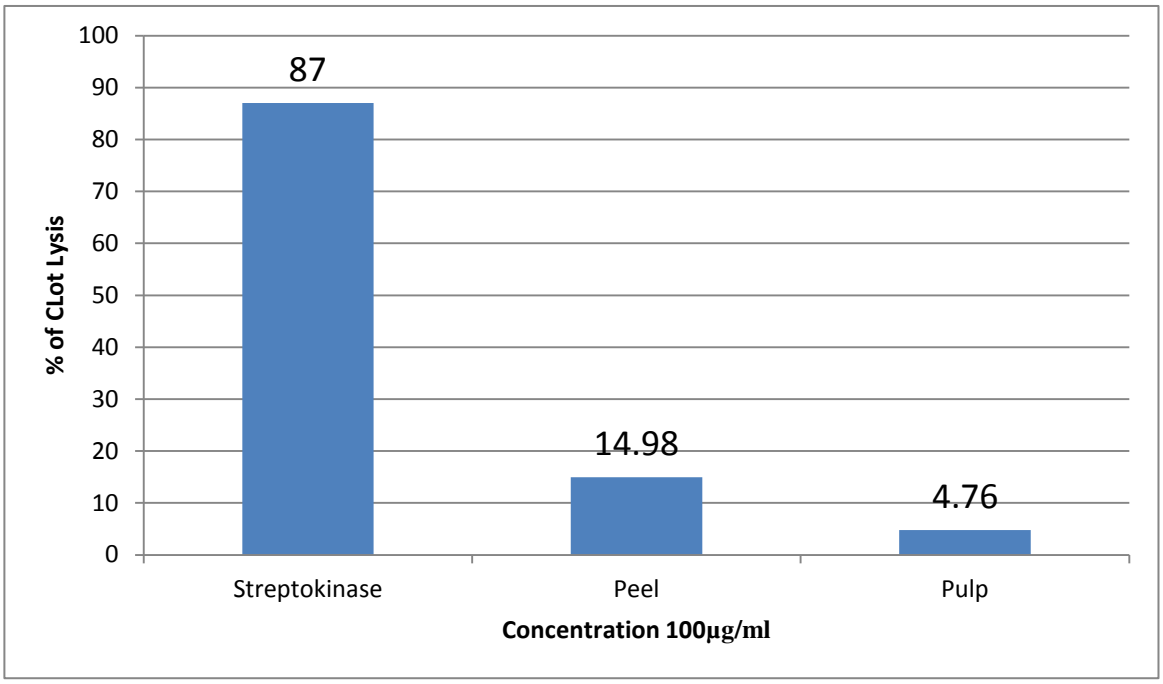

Fig 4.11 : Determination of Thrombolytic Activity (CME)

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 macroptera* was used in this test. From the table of result, the crude methanolic extract of *Citrus macroptera* (Peel) in concentration of 300µg/µl is 0.244 and for which Gallic acid equivalent per gram (GAE/gm of dried sample) of dried sample is 14.64. The pulp of *Citrus macroptera* crude methanolic extract gives absorbance of 0.345 on the same concentration and GAE/gm of dried sample is 20.70. Dried extracts of pulpof *Citrus macroptera* is a prominent source of phenolic compounds as 300µg/µl CME contain 20.70GAE/gm compared to the peel of the species.

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 the peel of *Citrus macroptera* gives absorbance of 0.0.024 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 of the pulp of *Citrus macroptera* has given absorbance of 0.029 and CE/gm of dried sample is 1.74. From the result, the pulp of *Citrus macroptera* gives highest GAE/gm of dried sample among the two parts tested. That means dried extracts of peel of *Citrus macroptera* is not a prominent source of flavonoid compounds. So dried extracts of peel of *Citrus macroptera* 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 the peel of *Citrus macroptera* crude methanolic extract gives absorbance of 0.098 in concentration of 300µg/µl for which Gallic acid equivalent /gm of dried sample (GAE/gm of dried sample) is 2.52. Crude methanolic extract of the pulp of *Citrus macroptera* gives

absorbance of 0.039 and GAE/gm of dried sample is 1.14. Here, the dried extracts of the peel of *Citrus macroptera* is representing that it has greater flavanol content thanthe dried extracts of pulp.

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, the peel extract of *Citrus macroptera* gives percentage of inhibition of12.00, 15.17, 17.42, 18.33for the concentration of 20 µl, 50 µl, 100µl, 200 µl respectively. The pulp extract of *Citrus macroptera* gives percentage of inhibition 7.92, 10.58, 15.50, 17.58 at the same concentrations mentioned before. Among the four different concentrations, 200 µg/µl concentrations of both the samples give highest radical scavenging activity. However, the peel extract of *Citrus macroptera* has shown the 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 he peel of *Citrus macroptera g*ives an absorbance of 0.082 in the concentration of 100µg/µl. The pulp of *Citrus macroptera in* the same concentration gives absorbance of 0.80. Among the two samples, the pulp extract of *C. macroptera* 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 the crude extracts because the 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 Acetyl Cholinesterase Inhibitory Activity:

The determination of Acetyl Cholinesterase inhibitory activity is done by Ellman's method. Acetyl cholinesterase inhibitory activity table presents that the peel extract of *Citrus macroptera* gives percentage of inhibition 27.37, 34.87, 49.88, 75.63for the concentrations of 20 µg/µl, 50 µg/µl, 100 µg/µl, 200µg/µl respectively. For the same concentrations the pulp

extract of *Citrus macroptera* gives percent of inhibition 21.63, 35.00, 41.63, 69.25. Donepezil (drug of choice) has been used as standard in this test which has percent of inhibition values of 78.34, 86.12, 91.44, 92.14.Among the two CME samples at a concentration of 200µg/µl the peel extract of *Citrus macroptera* gives the highest AchE inhibitory activity of 75.63% which is greater than the pulp extract. 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 Butyryl Cholinestserase Inhibitory Activity:

The determination of Butyryl cholinestserase inhibitory activity is done by Ellman's method. The table of Butyryl cholinestserase inhibitory activity represents that the peel extract of *Citrus macroptera* in the concentration of 20 µg/µl, 50 µg/µl, 100 µg/µl, 200µg/µl gives percent of inhibition of 6.43, 10.00, 12.86, 16.43. The pulp extract of *Citrus macroptera* gives percent of inhibition 8.43, 17.50, 18.72, 46.57 in the same concentrations respectively. Galantamine has been used as standard which has the percent of inhibition of 72.43, 86.17, 88.42, 91.41. The two CME samples have Butyryl cholinestserase inhibitory activity and with the increase of concentration the inhibitory activity is also increasing. In the 200µg/µl of concentration the pulp extract of *C. macroptera* gives the highest Butyryl cholinestserase inhibitory activityof 46.57% than the peel extract of the plant. 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 the peel extract of *Citrus macroptera* is 14.98 in the concentration of 100 µg/µl. In the same

concentration, the pulp extract of *Citrus macroptera* gives4.76 percent. Streptokinase has been used as standard which has clot lysis percentage of 87.01. Among the two CME samples the peel extract of *Citrus macroptera* has highest thrombolytic activity of 14.98% than the pulp extract. Comparing with Streptokinase standard the activity of the sample plants are much less because the purity of standard is higher than crude extracts while crude extracts contain many other agonistic or antagonistic compounds. So, there is a chance to form more active molecules from those plants.

5.9. Conclusion

Citrus macroptera contains phenolic, flavonoid and flavonol contents in high amount. The CME of peel and pulp were active in all the tests but among them, the CME of peel was found more active. The CME of peel was found more active in the test for total flavonoids and total flavonol content. The peel extract also showed more activity than the pulp extract in DPPH free radical scavenging activity test. The peel also showed high activity in Acetyl cholinesterase inhibitory test and thrombolytic test. Whereas the pulp extract was found more active in test for phenolic compounds and the test for reducing power. It has also showed activity in Butyryl cholinesterase inhibitory test. Thus, *C. macroptera* has potential antioxidant, cholinesterase inhibitory and thrombolytic activity. Although the plant showed high activity in the tests mentioned, further study should be done.

6.1. References :

Ahmad, I., Rahman, M., Rahman, M.M., Alam, M.M.M. and Hussain, M.S., 2013. Effect of Gamma Radiation on the Titrable Acidity and Vitamin C Content of Citrus Fruits. *International Journal of Agricultural Research, Innovation and Technology*, *2*(1), pp.1-6.

Alz.org. 2016. *Alzheimer's Disease & Dementia | Alzheimer's Association*. [online] Available at: http://www.alz.org/alzheimers_disease_what_is_ alzheimers.asp [Accessed 12 Dec. 2016].

Chutia, M., Bhuyan, P.D., Pathak, M.G., Sarma, T.C. and Boruah, P., 2009. Antifungal activity and chemical composition of Citrus reticulata Blanco essential oil against phytopathogens from North East India. *LWT-Food Science and Technology*, *42*(3), pp.777-780.

Conforti, F., Statti, G.A., Tundis, R., Loizzo, M.R. and Menichini, F., 2007. In vitro activities of Citrus medica L. cv. Diamante (Diamante citron) relevant to treatment of diabetes and Alzheimer's disease. *Phytotherapy Research*,*21*(5), pp.427-433

Dang, N.H., Nhung, P.H., Mai Anh, B.T., Thu Thuy, D.T., Minh, C.V. and Dat, N.T., 2016. Chemical Composition and α-Glucosidase Inhibitory Activity of Vietnamese Citrus Peels Essential Oils. *Journal of Chemistry*.

Dubois, B., Feldman, H.H., Jacova, C., Cummings, J.L., DeKosky, S.T., Barberger-Gateau, P., Delacourte, A., Frisoni, G., Fox, N.C., Galasko, D. and Gauthier, S., 2010. Revising the definition of Alzheimer's disease: a new lexicon. *The Lancet Neurology*, *9*(11), pp.1118-1127.

Gaillard, E., Muyard, F., Bevalot, F., Regnier, A. and Vaquette, J., 1994, December. Rutacea from New Caledonia. Chemical composition of stem bark of Citrus macroptera (Montr_ In *Annales pharma ceutiques francaises*; Vol. 53, No. 2, pp. 75-78.

Google Books., 2016. *Citrus Genetics, Breeding and Biotechnology*. [online] Available at: https://books.google.com.bd/books

Hynniewta, M., Malik, S.K. and Rao, S.R., 2011. Karyological studies in ten species of Citrus (Linnaeus, 1753)(Rutaceae) of North-East India. *Comparative cytogenetics*, *5*(4), p.277.

Jivad, N. and Rabiei, Z., 2014. A review study on medicinal plants used in the treatment of learning and memory impairments. *Asian Pacific Journal of Tropical Biomedicine*, *4*(10), pp.780-789.

Jousse, J., 2016. *Citrus Pages / Papedas*. [online] Citruspages.free.fr. Available at: http://citruspages.free.fr/papedas.html [Accessed 12 Dec. 2016].

Jstor.org., 2016. *On the History and Origin of Citrus on JSTOR*. [online] Available at: http://www.jstor.org/stable/2484763?seq=1#page_scan_tab_ contents [Accessed 12 Dec. 2016].

Kaviya, S., Santhanalakshmi, J., Viswanathan, B., Muthumary, J. and Srinivasan, K., 2011. Biosynthesis of silver nanoparticles using Citrus sinensispeel extract and its antibacterial activity. *SpectrochimicaActa Part A: Molecular and Biomolecular Spectroscopy*, *79*(3), pp.594-598.

Loizzo, M.R., Tundis, R., Bonesi, M., Menichini, F., De Luca, D., Colica, C. and Menichini, F., 2012. Evaluation of Citrus aurantifolia peel and leaves extracts for their chemical composition, antioxidant and anti- cholinesterase activities. *Journal of the Science of Food and Agriculture*, *92*(15), pp.2960-2967.

Lopes Campêlo, L.M., Gonçalves e Sá, C., de Almeida, A.A.C., Pereira da Costa, J., Costa Marques, T.H., Mendes Feitosa, C., Barros Saldanha, G. and Mendes de Freitas, R., 2011. Sedative, anxiolytic and antidepressant activities of Citrus limon (Burn) essential oil in mice. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, *66*(8), pp.623-627.

Lota, M.L., de Rocca Serra, D., Tomi, F., Bessiere, J.M. and Casanova, J., 1999. Chemical composition of peel and leaf essential oils of Citrus medica L. and C. limonimedica Lush. *Flavour and fragrance journal*, *14*(3), pp.161-166.

Lota, M.L., de Rocca Serra, D., Tomi, F. and Casanova, J., 2000. Chemical variability of peel and leaf essential oils of mandarins from Citrus reticulata Blanco. *Biochemical Systematics and Ecology*, *28*(1), pp.61-78.

M. N. Miah, Sahina Islam, and Syed Hadiuzzaman 2002. Regeneration of plantlets through somatic embryogenesis from nucellus tissue of *Citrus macroptera* Mont. var. *anammensis* ('Sat Kara'). *Plant Tissue Culture and Biotechnology* 12(2), pp.167-172

Malik, S.K. and Chaudhury, R., 2006. The cryopreservation of embryonic axes of two wild and endangered Citrus species. *Plant genetic resources: characterization and utilization*, *4*(03), pp.204-209.

Manipursfac.com., 2016. [online] Available at: http://manipursfac.com/wild-orange-citrus-macroptera/ [Accessed 12 Dec. 2016].

Marak, C.K. and Laskar, M.A., 2010. Analysis of phenetic relationship between Citrus indica Tanaka and a few commercially important citrus species by ISSR markers. *Scientiahorticulturae*, *124*(3), pp.345-348.

Menichini, F., Loizzo, M.R., Bonesi, M., Conforti, F., De Luca, D., Statti, G.A., de Cindio, B., Menichini, F. and Tundis, R., 2011. Phytochemical profile, antioxidant, anti-inflammatory and hypoglycemic potential of hydroalcoholic extracts from Citrus medica L. cv Diamante flowers, leaves and fruits at two maturity stages. *Food and Chemical Toxicology*, *49*(7), pp.1549-1555.

Miah, M.N., Islam, S. and Hadiuzzaman, S., 2002. Regeneration of plantlets through somatic embryogenesis from nucellus tissues of Citrus macroptera Mont. var. anammensis ('Sat Kara'). *Plant Tissue Cult*, *12*, pp.167-172.

Panara, K., 2012. A review on phytochemical and pharmacological properties of Citrus medica Linn. *International Journal of Pharmaceutical & Biological Archive*, *3*(6).

Patil, J.R., Jayaprakasha, G.K., Murthy, K.C., Chetti, M.B. and Patil, B.S., 2010. Characterization of Citrus aurantifolia bioactive compounds and their inhibition of human pancreatic cancer cells through apoptosis. *Microchemical Journal*, *94*(2), pp.108-117.

Plants.usda.gov., 2016. *Plants Profile for Citrus macroptera*. [online] Available at: http://plants.usda.gov/core/profile?symbol=CIMA10 [Accessed 12 Dec. 2016].

Paul, S., Hossen, M.S., Tanvir, E.M., Islam, M.A., Afroz, R., Ahmmed, I., Saha, M., Gan, S.H. and Khalil, M.I., 2015. Antioxidant properties of Citrus macroptera fruit and its in vivo effects on the liver, kidney and pancreas in wistar rats. *International Journal of Pharmacology*, *11*(8), pp.899-909.

Paul, S., Islam, M.A., Tanvir, E.M., Ahmed, R., Das, S., Rumpa, N.E., Hossen, M.S., Parvez, M., Gan, S.H. and Khalil, M.I., 2016. Satkara (Citrus macroptera) Fruit Protects against Acetaminophen-Induced Hepatorenal Toxicity in Rats. *Evidence-Based Complementary and Alternative Medicine*.

Rahman, H., Eswaraiah, M.C. and Dutta, A., 2014. Neuropharmacological activities of ethanolic extract of Citrus macroptera (Varannamensis) fruit peels. *Global Journal of Pharmacology*, *8*(4), pp.609-616.

Rana, V.S. and Blazquez, M.A., 2012. Compositions of the volatile oils of Citrus macroptera and C. maxima. *Natural product communications*, *7*(10), pp.1371-1372.

Rao, R. V., Descamps, O., John, V., &Bredesen, D. E., 2012. Ayurvedic medicinal plants for Alzheimer's disease: a review. *Alzheimer's Research & Therapy*, *4*(3), 22.

Rekha, C., Poornima, G., Manasa, M., Abhipsa, V., Devi, P.J., Kumar, V.H.T. and Kekuda, P.T.R., 2012. Ascorbic acid, total phenol content and antioxidant activity of fresh juices of four ripe and unripe citrus fruits. *Chemical Science Transactions*, *1*(2), pp.303-310.

Sidana, J., Saini, V., Dahiya, S., Nain, P. and Bala, S., 2013. A Review on Citrus-"The Boon of Nature". *Int. J. Pharm. Sci. Rev. Res*, *18*, pp.20-27.

Singh, P., Shukla, R., Prakash, B., Kumar, A., Singh, S., Mishra, P.K. and Dubey, N.K., 2010. Chemical profile, antifungal, antiaflatoxigenic and antioxidant activity of Citrus maxima Burm. and Citrus sinensis (L.) Osbeck essential oils and their cyclic monoterpene, DL-limonene. *Food and Chemical Toxicology*, *48*(6), pp.1734-1740.

Singhal, A.K., Naithani, V. and Bangar, O.P., 2012. Medicinal plants with a potential to treat Alzheimer and associated symptoms. *International Journal of Nutrition, Pharmacology, Neurological Diseases*, *2*(2), p.84.

Sood, S., Bansal, S., Muthuraman, A., Gill, N.S. and Bali, M., 2009. Therapeutic potential of Citrus medica L. peel extract in carrageenan induced inflammatory pain in rat. *Research Journal of Medicinal Plant*, *3*(4), pp.123-133.

Uddin N, Hasan MR, Hasan MM, Hossain MM, Alam MR, Hasan MR, et al., 2014. Assessment of Toxic Effects of the Methanol Extract of *Citrus macroptera* Montr. Fruit via Biochemical and Hematological Evaluation in Female Sprague-Dawley Rats. PLoS ONE 9(11): e111101. doi:10.1371/journal.pone.0111101

Uddin, N., Hasan, M.R., Hossain, M.M., Sarker, A., Hasan, A.N., Islam, A.M., Chowdhury, M.M.H. and Rana, M.S., 2014. In vitro α–amylase inhibitory activity and in vivo hypoglycemic effect of methanol extract of Citrus macroptera Montr. fruit. *Asian Pacific journal of tropical biomedicine*, *4*(6), pp.473-479.

Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J. and Pérez-Álvarez, J., 2008. Antifungal activity of lemon (*Citrus lemon L.*), mandarin (*Citrus reticulata L.*), grapefruit (*Citrus paradisi L.*) and orange (*Citrus sinensis L.*) essential oils. *Food control*, *19*(12), pp.1130-1138.

Waikedre, J., Dugay, A., Barrachina, I., Herrenknecht, C., Cabalion, P. and Fournet, A., 2010. Chemical composition and antimicrobial activity of the essential oils from New Caledonian Citrus macroptera and Citrus hystrix. *Chemistry& biodiversity*, *7*(4), pp.871-877.