In-Vitro Evaluation of Cholinesterase Inhibitory and Antioxidant Activities of *Tamarindus indica* for the Treatment of Alzheimer's Disease

A dissertation submitted to the
Department of Pharmacy, East West University
in the partial fulfillment for the degree of Bachelor of Pharmacy.

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Declaration by the Research Candidate

I, Md. Ashiqur Rahman, ID: 2012-3-70-030, hereby declare that the dissertation entitled "*In-vitro* evaluation of cholinesterase inhibitory and antioxidant activities of *Tamarindus indica* for the treatment of alzheimer's disease" submitted by me to the Department of Pharmacy, East West University and in the partial fulfillment of the requirement of the degree of Bachelor of Pharmacy, under the supervision and guidance Kusal Biswas, Lecturer, Department of Pharmacy, East West University.

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Certificate by the Chairperson

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Serial no.	Abbreviation	Full form
1.	ACh	Acetylcholine
2.	AChE	Acetylcholinesterase
3.	AchIs	Acetylcholinesterase inhibitors
4.	AD	Alzheimer's disease
5.	ApoE	Apolipoprotein E
6.	ApoE2	Apolipoprotein E2
7.	ApoE3	Apolipoprotein E3
8.	ApoE4	Apolipoprotein E4
9.	APP	Amyloid precursor protein
10.	APP	Amyloid precursor protein
11.	ATP	Adenosine triphosphate
12.	Aβ	Amyloid beta
13.	AβPP	Amyloid beta Protein Precursor
14.	BACE	β-site cleaving enzyme
15.	BCA	Bicinchoninic acid
16.	BHT	Butylated hydroxy toluene
17.	BSA	Bovine serum albumin
18.	BuChE	Butyrylcholinesterase
19.	C99	C-terminal fragment
20.	CE	Catechin Equivalent
21.	CME	Crude methanol extract
22.	CLF	Chloroform fraction
23	CNS	Central nervous system
24	Conc.	Concentration
25.	DNA	Deoxyribonucleic acid
26.	DPPH	1, 1-diphenyl-2-picrylhydrazyl
27.	DTNB	5, 5-dithio-bis-(2-nitro) benzoic acid
28.	EAF	Ethyl acetate fraction
29.	EDTA	Ethylene diamine tetraacetic acid
30.	FCR	Folin Ciocalteu Reagent
31.	FDA	Food and drug administration

32.	$FeCl_3$	Ferric chloride
33.	$FeSO_4$	Ferrous sulphate
34.	GAE	Gallic acid equivalent
35.	gm	Gram
36.	H_2O_2	Hydrogen peroxide
37.	HNE	4-hydroxy-2-nonenal
38.	IC_{50}	**Conc.** required for 50% Inhibition
39.	K_2HPO_4	Dipotassium hydrogen phosphate
40.	$K_3Fe\ (CN)_6$	Potassium ferricyanide
41.	KH_2PO_4	Monopotassium hydrogen phosphate
42.	KOH	Potassium hydroxide
43.	*L. globosus*	*Loranthus globosus*
44.	MCI	Mild cognitive impairment
45.	MDA	Malondialdehyde
46.	mg	Milligram
47.	ml	Milliliter
48.	mM	Millimolar
49.	MTB	Methylene blue
50.	MTHFR	**5,10**-methylenetetrahydrofolate reductase
51.	nAChRs	Neuronal acetylcholine receptors
52.	NaCl	Sodium chloride
53.	NCEs	New Chemical entities
54.	NFTs	Neurofibrillary tangles
55.	NMR	Neuclear Magnetic Resonance
56.	nm	Nanometer
57.	NMDA	N-methyl- D -aspartate
58.	NPs	Neuritic plaques
59.	PEF	Petrolieum ether fraction
60.	PS-1	Presenilin-1
61.	PS-2	Presenilin-2
62.	SAP	Serum Amyloid P component
63.	sAPPβ	soluble extracellular fragment of APP
64.	STD	Standard deviation
65.	TBA	Thiobarbituric acid

66.	TBARS	Thiobarbituric acid-reactive substance
67.	TCA	Trichloro acetic acid
68.	TLC	Thin Layer Chromatography
69.	UV	Ultraviolet
70.	α	Alpha
71.	β	Beta
72.	γ	Gamma
73.	µl	Micro liter
74.	µg	Micro gram

Abstract

Oxidative stress and low amount of neurotransmitter specially acetylcholine are main characteristics of Alzheimer's disease (AD), which is a progressive neuro-degenerative disease. Prolonging the function of acetylcholine by inhibiting acetylcholinesterase or butyrylcholinesterase enzyme and reducing oxidative stress with antioxidants are effective treatment therapy of AD. *Tamarindus indica* is a well-known medicine plant with a variety of medicinal uses. In this study we examined cholinesterase inhibitory activity and antioxidant activity of tamarind leaf and bark extract. Cholinesterase inhibitory activity with IC50 of 188.67 µg/ml and 176.30 µg/ml for acetylcholinesterase and butyrylcholinesterase enzyme respectively suggested that it possesses a good source of enzyme inhibitor. In conclusion, we can say *Tamarindus indica* provide not only Cholinesterase inhibitory activity but also antioxidant activity which can be useful for further research to get potential molecule from it.

Chapter 1

Introduction

INTRODUCTION

1.1. General Introduction

Medicine is the wonder of the world and blessings for mankind. From the very ancient time men used various plant parts as their wound healing. Pharmacy, the science and practice of medicine and its primary source plays an important role in identifying the new molecule of drug through both synthetically and from that of natural origin. The history of drug from natural sources is very significant and well known. By trial and error, primitive man must have acquired knowledge that was useful in determining which plants and animals possessed food value and which were to be avoided because they were unpalatable, poisonous, or dangerous. (Gani, 1998)

Medicinal plants continue to be an important therapeutic aid for alleviating ailments of humankind. Search for eternal health and longevity and to seek remedy to relieve pain and discomfort prompted the early man to explore his immediate natural surrounding and tried many plants, animal products and minerals and developed a variety of therapeutic agents. Over millennia that followed the effective agents amongst them were selected by the process of trial, error, empirical reasoning and even by experimentation. These efforts have gone in history by the name discovery of 'medicine'. In many eastern cultures such as those of India, China and the Arab/Persian world this experience was systematically recorded and incorporated into regular system of medicine that refined and developed and became a part of the Materia Medica of these countries. The ancient civilization of India, China, Greece, Arab and other countries of the world developed their systems of medicine independent of each other but all of them were predominantly plant based. But the theoretical foundation and the insights and in depth understanding on the practice of medicine that we find in Ayurveda is much superior among organized ancient systems of medicine. According to past records, Babylonians were aware of a large number of medicinal plants and their properties. Some of the plants are still used today in the same way and for the same purposes. The earliest mention of the medicinal use of plants in the Indian subcontinent is found in the Rig Veda. Which noted that the Indo-Aryans used the Soma plant (Amanita muscaria), a narcotic and hallucinogenic mushroom, as a medicinal agent. The Vedas made many references to the healing powers of sharpagandha (Rauvolfia serpentina), while a comprehensive Indian herbal book, the Charaka Samhita, cites more than 500 medicinal plants.(Samsad 2004)

1.2. Plants in traditional medicine

It is estimated that 70-80% of people worldwide rely chiefly on traditional, largely herbal medicine to meet their primary healthcare needs. (Fansworth, 1991) The global demand for herbal medicine is not only large, but growing. The market for Ayurvedic medicines is estimated to be expanding at 20% annually in India, while the quantity of medicinal plants obtained from just one province of China (Yunnan) has grown by 10 times in the last 10

years. (Shengji, 2001) An example of increased pressure on collecting grounds is provided by the Gori valley in the Indian Himalayas, where the annual period of MAP harvesting has increased from 2 to 5 months. (Lambert et. al., 1997) Factors contributing to the growth in demand for traditional medicine include the increasing human population and the frequently inadequate provision of Western (allopathic) medicine in developing countries.

1.3.Status of medicinal plants in Bangladesh

About 500 medicinal plants have been reported to occur in Bangladesh. The local people conserve traditional knowledge through their experience and practices, which is handed down orally without any documentation. The over exploitation of wild medicinal plants has become a threat to its extinction. In Bangladesh there is no systematic cultivation process of conservation strategies about medicinal plants. There is no government policy or rules and regulations about the medicinal plants cultivation conservation and marketing. There are almost 422 herbal medicinal companies using medicinal plants as raw materials mostly by importing from abroad.(Khan, 1975)

Table 1.1 Small description of some of the medicinal plants of Bangladesh:

Aloe Vera Scientific Name: Aloe barbadensis Family: Liliaceae Used Part: Whole plant Medicinal Use: Purgative, anthelmintic, carminative and digestive.	 (Smartcooky, 2016)
Arjun Scientific Name: Termilnalia arjuna Family: Combretaceae Used part: Bark, leaf Medicinal use: Used on diarrhea, angia	 (Ayurveda-foryou, 2016)
Bastard Myrobalan Scientific Name: Terminallia belerica Family: Combretaceae Used part: Leaf, seed Medicinal use: Used in cancer, cardiac disease	 (Youtube, 2016)

Bay leaf Scientific Name: Cinnamomum tamala Family: Lauraceae Used Part: Leaf Medicinal Use: Carminative, used in sore throat, diarrhea	 (DeLallo Foods, 2016)
Bean Scientific Name: Dolichos lablab Family: Leguminosae Used part: Seed Medicinal use: Used in malabsorption, diarrhea	 (Annie's "Back To Eden" Garden, 2015)
Betel Nut Scientific Name: Areca catechu Family: Palmae Used part: Seed Medicinal use: Antimicrobial, used in abdominal pain, edema	 (Simonsblogpark, 2016)
Black berry Scientific Name: Syzygium cumini Family: Myrtaceae Used Part: Whole plant Medicinal Use: Sore throat, bronchitis, asthma, dysentery	 (Plantsguru, 2014)
Black Pepper Scientific Name: Piper nigrum Family: Piperaceae Used part: Dried unripe fruit Medicinal use: Epilepsy, chronic bronchitis, asthma	 (BBC, 2016)

Kalmegh Scientific Name: Andrographis paniculata Family: Acathacea Used part: Whole plant Medicinal Use: Gastric, fever, liver diseases, dysentery, metabolic problems	 (101 Herbs, 2016)
Custard Apple Scientific Name: Annona squamosa Family: Annonaceae Used Part: Fruit, leaf Medicinal Use: Jaundice, laxative, anthelmintic	 (Sunrise, 2016)
Ginger Scientific Name: Zinger officinale Family: Zingiberaceae Used part: Root Medicinal use: Antitussive	 (Serious Eats, 2016)
Henna Scientific Name: Lawsonia inermis Family: Lythraceae Used Part: Leaf, bark, seed Medicinal Use: Jaundice, enlarged spleen, some skin disease.	 (Perikali, 2011)
Elephant Apple Scientific Name: Dillenia indica Family: Dilleniaceae Used part: Fruit, leaf, bark Medicinal use: Expectorant, antifungal, antibacterial	 (Snaplant, 2016)

Fenugreek Scientific Name: Trigonella foenum-graecum Family: Fabaceae Used Part: Whole plant Medicinal Use: Chronoc cough, antipyretic, carminative, anthelmintic, antringent to the bowel, reduce enlargement of liver and spleen	 (Pramoda, 2016)
Indian gooseberry Scientific Name: Phyllanthus emblica Family:Euphorbiaceae Used part: Fruit Medicinal use: Antiinflammatory	 (Womentribe, 2016)
Indian pennywort Scientific Name: Centella asiatica Family: Apiaceae Used part: Whole plant Medicinal use: Antiprotozoal, diuretic, used in leprosy, eczema	 (Youtube, 2016)
Indian Sarsaparilla Scientific Name: Hemidesmus indicus Family: Asclepiadaceae Used Part: Root Medicinal Use: Blood purifier, demulcent, diuretic, purgative	 (Goldenpoppyherb, 2016)
Keshraj Scientific Name: Eclipta prostate Family: Compositae Used part: Whole plant Medicinal use: Uterine bleeding, premature graying of hair	 (Lonely Traveller, 2016)

Malabar Nut Scientific Name: Adhatoda zeylanica Family: Acanthaceae Used Part: Bark, root, leaf Medicinal Use: Expectorant, antirheumatic	 (Flowerpictures, 2016)
Neem Scientific Name: Azadirachta indica Family: Meliaceae Used Part: Leaf Medicinal Use: Anthelmintic, Antiulcerant, antiinflammation, fever, skin disease	 (Artemisinine, 2016)
Oriental arborvitae Scientific Name: Thuju Orientalis Family: Cupressaceae Used part: Leaf, seed Medicinal use: GIT bleeding, alopecia, constipation,insomnia	 (Washington State University, 2016)
Papaya Scientific Name: Carica papaya Family: Cariaceae Used Part: Fruit, leaf, seed Medicinal Use: Digestive, anthelmintic	 (Perfectimage, 2016)
Pine Apple Scientific Name: Ananas sativus Family: Bromeliaceae Used Part: Leaf Medicinal Use: Anthemmintic	 (Freenology, 2016)

Pomegranate Scientific Name: Punica granatum Family: Punicacaea Used Part: Fruit, bark, seed Medicinal Use: Strengthen gums, cardiac tonic	 (Turinitalyguide, 2016)
Wood Apple Scientific Name: Aegle Marmelos Family: Rutaceae Used part: Fruit Medicinal Use: Dysentery, diarrhea	 (123RF, 2016)

1.4.Medicinal Plants in World Market

The largest global markets for MAPs are China, France, Germany, Italy, Japan, Spain, the UK and the US. Japan has the highest per capita consumption of botanical medicines in the world (Laird, 1999). The International Council for Medicinal and Aromatic Plants expects world growth during 2001 and 2002 to be approximately 8-10 per cent a year. (Srivastava, 2000). In 1999, the world market for herbal remedies was US$19.4 billion, with Europe in the lead (US$6.7 billion), followed by Asia (US$5.1 billion), North America (US$4.0 billion), Japan (US$2.2 billion) and the rest of the world (US$1.4 billion) (Laird, 2002). India is a major exporter of raw MAPs and processed plant-based drugs. Exports of crude drugs from India in 1994-95 were valued at US$53,219 million and of essential oils US$13,250 million. (Lambert et. al., 1997). Overall sales of botanical medicine products in China in 1995 were estimated at US$5 billion. The botanical medicine market in Japan in 1996 was estimated at US$2.4 billion.

1.5.Herbal drug research: Bioactivity guided approach

Books on herbal medicinal practice report numerous medicinal plants, which are still not investigated. These plants can be subjected to pharmacologic screening as per their traditional use to evaluate their utility. In case of significant result, chromatographic and spectroscopic methods can be applied to isolate the responsible agent. Bioactivity guided approach has three characteristic phases of investigation.

Firstly**,** Biological activity is detected in crude material, and a bioassay system is set up to permit the identification of active fractions and discarding the inactive ones.
Secondly, the crude material is fractionated by the most appropriate chemical procedures, all fractions are tested, and active fractions are further fractionated, and so on, until pure compounds are obtained.
Thirdly, the chemical structures of pure compounds are determined. (Goldstein, 1974)

1.6.Approaches to new product discovery

This subject is covered authoritatively in some recent publications (Kate, 1999)and partially elsewhere in this paper. Several stages are involved in the process of prospecting the chemical properties of plants to discover drugs or other novel products. First, unless discoveries are fortuitous, decisions are made about which plants to sample and how to sample them. Sampling may be in the field or from ex situ collections, the latter perhaps represented by plants growing in botanical gardens or by dried specimens in herbaria. These decisions are based on published and unpublished information, including sometimes knowledge of local medical uses and about the relative difficulty of undertaking research in different contexts. The next step involves isolation of chemical fractions for automated screening, for example the in vitro testing of activity against cell lines. Promising results may lead to further tests, including perhaps clinical trials, and these may result in the development, including licensing, of marketable products. As an alternative to chemical screening, there is growing interest today in screening extract from plants for genetic information, a branch of science set to grow spectacularly (Hamilton, 2003)

Traditional practitioner dispensing his own medicines is being gradually shifted to herbal drug stores which are profit-oriented. As a result, there is no guarantee of the authenticity and quantity of plant material used in the preparations. The qualities of traditional medicines so produced vary widely and may not even be effective. Therefore, there is a need to select proper and appropriate technologies for the industrial production of traditional medicines such that the effectiveness of the preparation is maintained. Traditional methods used have many disadvantages which could be corrected by selecting the suitable technologies. It has to be stated that the traditional methods were dependent on the status of technology that was available at that time. It therefore follows that these can be modified and improved using the technologies available today to make them more effective, stable, reproducible, controlled and in dosage forms that can easily be transported or taken to office.

Hence the introduction of appropriate, simple and low-cost technologies should be encouraged maintaining as much as possible the labor-intensive nature of such activities, conservation of biodiversity through small-scale production and preservation of cultural knowledge. Use of sophisticated modern technology will alienate the traditional practitioners

as he has no control over such production methods. Even in the use of appropriate technologies, the practitioner who produces these drugs has to be educated about the advantages of using such production and quality control methods. One major concern in introducing modern technology for the production of traditional medicines is whether the final preparation will be acceptable to the practitioner who has sole faith in extemporaneous preparations. This problem has to be overcome by a process of education, whereby the disadvantage of the old methods and the advantage of the new methods can be imparted. The value of medicinal plant as a source of foreign exchange for developing countries depends on the use of those plants as raw materials in the pharmaceutical industry. These raw materials are used to:

- Isolate pure active compounds for formulation into drugs (guinini, reserpine, digoxin etc.)
- Isolate intermediates for the production of semi-synthetic drugs.
- Prepare standardized galenicals (abstracts, powders, tinctures etc.) If one is to produce known pure phytopharmaceutical used in modern medicine more processing stages and more sophisticated machinery are required.

Furthermore safety and pollution aspects have to be considered. Certain plants are rich sources of intermediates used in the production of drugs. The primary processing of parts of plants containing the intermediate could be carried out in the country of origin thus retaining some value of the resource material. Processed products (galenicals) from plants could be standardized fluid/ solid extract or powders or tinctures. Standardized extract of many plants are widely used in health care. Some of these have to be formulated for incorporation in modern dosage forms. New formulations require some development work, particularly on account of the nature of the processed products. Plant extract are difficult to granulate, sensitive to moisture and prone to microbial contamination. Hence the types of excipients to be used and the processing parameters have to be determined. (Planning commission, India, 2000)

1.7 Alzheimer's Disease:

Alzheimer's disease (AD) is a neurodegenerative disordercharacterized by a progressive decline of memory andcognition. Amyloid-β (βA), neurofibrillary tangles (NFT)and synaptic loss; particularly, the deficiency of acetylcholine(ACh) and the degeneration of cholinergic neuronsin the cortex and hippocampus, nucleus basalis ofMeynert, are the hallmarks of AD. A loss of ACh isconsidered to play a vital role in the learning and memory deterioration of AD patients.

Acetylcholine is an organic molecule liberated at nerve endings as a neurotransmitter. It is produced by the synthetic enzyme cholineacetyl-transferase which uses acetyl coenzyme-A andcholine as substrates for the formation of acetylcholinein specific cells known as cholinergic neurons. Neurotransmitterdisturbances and insufficient cholinergicfunctions are identified among the pathological features in central nervous system disorders.

The United Nations estimate that the number of people suffering from age-related neurodegeneration, particularly from AD, will exponentially increase from 25.5 million in 2000 to an estimated 114 million in 2050. According to preliminary data from CDC (Centers for Disease Control and Prevention), in 2006, approximately 72,914 deaths were recorded as being caused by AD. (Fransworth, 1991). An estimated 5 million people in the US suffer with over $70 billion in expenses annually. The data emphasizes the need for extensive research on AD.

Table 1.2: Common types of dementia and their characteristics:

Type of Dementia	Characteristics
Alzheimer's disease	♠ Most common type of dementia; accounts for an estimated 60 to 80 percent of cases. ♠ Difficulty remembering names and recent events is often an early clinical symptom; apathy and depressionare also often early symptoms. Later symptoms include impaired judgment, disorientation, confusion, behaviorchanges and difficulty speaking, swallowing and walking. ♠ New criteria and guidelines for diagnosing Alzheimer's were proposed in 2011. They recommend that Alzheimer'sdisease be considered a disease that begins well before the development of symptoms. ♠ Hallmark brain abnormalities are deposits of the protein fragment beta-amyloid (plaques) and twisted strands ofthe protein tau (tangles) as well as evidence of nerve cell damage and death in the brain.
Vascular dementia	♠ Previously known as multi-infarct or post-stroke dementia, vascular dementia is less common as a sole causeof dementia than is Alzheimer's disease. ♠ Impaired judgment or ability to make plans is more likely to be the initial symptom, as opposed to the memoryloss often associated with the initial symptoms of Alzheimer's. ♠ Vascular dementia occurs because of brain injuries such as microscopic bleeding and blood vessel blockage. ♠ The location of the brain injury determines how the individual's thinking and physical functioningare affected. ♠ In the past, evidence of vascular dementia was used to exclude a diagnosis of Alzheimer's disease (and viceversa). That practice is no longer considered consistent with pathologic evidence, which shows that the brainchanges of both types of dementia can be present simultaneously. When any two or more types of dementia are present at the same time, the individual is considered to have "mixed dementia."

Dementia with Lewy bodies (DLB*)*	• People with DLB have some of the symptoms common in Alzheimer's, but are more likely than people with Alzheimer's to have initial or early symptoms such as sleep disturbances, well-formed visual hallucinations,and muscle rigidity or other parkinsonian movement features. • Lewy bodies are abnormal aggregations (or clumps) of the protein alpha-synuclein. When they develop ina part of the brain called the cortex, dementia can result. Alpha-synuclein also aggregates in the brains ofpeople with Parkinson's disease, but the aggregates may appear in a pattern that is different from DLB. • The brain changes of DLB alone can cause dementia, or they can be present at the same time as the brainchanges of Alzheimer's disease and/or vascular dementia, with each entity contributing to the developmentof dementia. When this happens, the individual is said to have "mixed dementia."
Frontotemporal lobar degeneration (FTLD)	• Includes dementias such as behavioral variant FTLD, primary progressive aphasia, Pick's disease andprogressive supranuclear palsy. • Typical symptoms include changes in personality and behavior and difficulty with language. • Nerve cells in the front and side regions of the brain are especially affected. No distinguishing microscopicabnormality is linked to all cases. • The brain changes of behavioral variant FTLD may be present at the same time as the brain changes ofAlzheimer's, but people with behavioral variant FTLD generally develop symptoms at a younger age(at about age 60) and survive for fewer years than those with Alzheimer's.
Mixed dementia	• Characterized by the hallmark abnormalities of Alzheimer's and another type of dementia — most commonly,vascular dementia, but also other types, such as DLB. • Recent studies suggest that mixed dementia is more common than previously thought.
Parkinson's disease	• As Parkinson's disease progresses, it often results in a severe dementia similar to DLB or Alzheimer's. • Problems with movement are a common symptom early in the disease. • Alpha-synuclein aggregates are likely to begin in an area deep in the brain called the substantianigra. Theaggregates are thought to cause degeneration of the nerve cells that produce dopamine. • The incidence of Parkinson's disease is about one-tenth that of Alzheimer's disease.
Creutzfeldt-Jakob disease	• Rapidly fatal disorder that impairs memory and coordination and causes behavior changes. • Results from an infectious misfolded protein (prion) that causes other proteins throughout the brain tomisfold and thus malfunction. • Variant Creutzfeldt-Jakob disease is believed to be caused by consumption of products from cattle affectedby mad cow disease.

Normal pressure hydrocephalus	♠ Symptoms include difficulty walking, memory loss and inability to control urination. ♠ Caused by the buildup of fluid in the brain. ♠ Can sometimes be corrected with surgical installation of a shunt in the brain to drain excess fluid.

1.7.1. Stages of AD:

The stages below provide a general idea of how abilities change during the course of the disease.

Stage 1: No impairment.

Stage 2: Very mild decline.

Stage 3: Mild decline.

Stage 4: Moderate decline.

Stage 5: Moderately severe decline.

Stage 6: Severe decline.

Stage 7: Very severe decline.

Not everyone will experience the same symptoms or progress at the same rate. This seven-stage framework is based on a system developed by Barry Reisberg, M.D., clinical director of the New York University School of Medicine's Silberstein Aging and Dementia Research Center.

Table 1.3: Stages and characteristics of AD:

Stages	Characteristics
Stage 1:	***No impairment (normal function)*** The person does not experience any memory problems. An interview with a medical professional does not show any evidence of symptoms of dementia.
Stage 2:	***Very mild cognitive decline (may be normal age-related changes or earliest signs of Alzheimer's disease)***

	The person may feel as if he or she is having memory lapses — forgetting familiar words or the location of everyday objects. But no symptoms of dementia can be detected during a medical examination or by friends, family or co-workers.
Stage 3:	***Early confusional (Mild Cognitive Decline).*** ***Duration - 2 to 7 years.*** Early-stage Alzheimer's is sometimes diagnosed at this stage. • The patient has slight difficulties which have some impact on certain everyday functions. In many cases the patient will try to conceal the problems. • Problems include difficulties with word recall, organization, planning, mislaying things, failing to remember recently learned data which may cause problems at work and at home - family members and close associates become aware. • Problem reading a passage and retaining information from it. • The ability to learn new things may be affected. • Problems with organization. • Moodiness, anxiety and in some cases depression.
Stage 4:	***Moderate Cognitive Decline. (Mild or Early Stage Alzheimer's Disease). Duration - about 2 years.*** With these symptoms diagnosis is easy to confirm. • Still identifies familiar people and is aware of self. • Reduced memory of personal history • Problems with numbers which impact on family finance - managing bills, checkbooks etc. Previously doable numerical exercises, such as counting backwards from 88 in lots of 6s become too difficult. • Knowledge of recent occasions or current events is decreased. • Sequential tasks become more difficult, including driving, cooking, planning dinner for guests, many domestic chores, shopping alone, and reading and then selecting what is in a menu at the restaurant. • Withdraws from conversations, social situations and mentally challenging situations. • Denies there is a problem and becomes defensive.

	• Requires help with some of the more complicated aspects of independent living.
Stage 5:	***Moderately Severe Cognitive Decline (Moderate or Mid-stage Alzheimer's Disease). Duration - about 18 months.*** • Cognitive deterioration is more serious. • Cannot survive independently in the community and requires some assistance with day-to-day activities. • Cannot remember details about personal history, such as name of where they went to school, telephone numbers, personal address, etc. • Confused about what day it is, month, year. • Confused about where they are or where things are. • Problems with numbers, mathematical abilities get worse. • Easy prey for scammers. Require supervision and sometimes help when dressing, including selecting right clothing for the season or occasion. • Require help carrying out some daily living tasks. • Can still eat and go to the toilet unaided. • Unable to recall current information consistently. • Usually remember substantial amounts about themselves, such as their name, name of spouse and children.
Stage 6:	***Severe Cognitive Decline (Moderately Severe Mid-stage Alzheimer's Disease).*** ***Duration - about 2½ years.*** Memory continues to deteriorate. There is a considerable change in personality. Require all-round help with daily activities. • Virtually totally unaware of present and most recent experiences. • Cannot recall personal history very well. • Can still usually recall their own name. • Know family members are familiar but cannot recall their names. • Can communicate pleasure and pain nonverbally. • Ability to dress progressively deteriorates.

	• Need help dressing and undressing. • Ability to bathe and wash self progressively deteriorates. • Fecal and/or urinary incontinence more likely. • Need help when going to the toilet - flushing, wiping, and disposing of tissues. • Disruption of sleep patterns. • Wander off and become lost. • Suspicious, • Paranoid and aggressive. • Patient may believe caregiver is an impostor, devious, scheming, cunning, dishonest. • Repeat words, phrases or repetitively utters sounds. • Repetitive/compulsive behavior, such as tearing up tissues or wringing hands.Disturbed, agitated, especially later on in the day. • Hallucinations, also more common later on in the day. May hear, smell or see things that are not there. • Eventually need care and supervision, but can respond to non-verbal stimuli.
Stage 7:	***Very Severe Cognitive Decline (Severe or Late-stage Alzheimer's Disease). Duration - 1 to 2½ years.*** During the last stage of Alzheimer's disease patients lose the ability to respond to their environment, they cannot speak and eventually cannot control movement. The duration of this stage may depend on the quality of care the patient receives. • Severely limited cognitive ability. • Patients lose their ability to recognize speech, but may utter short words or moans to communicate. • Usually the ability to walk unaided is lost first, then the ability to sit unaided, plus the ability to smile and eventually the ability to hold the head up. • Body systems start to fail and health deteriorates. • Swallowing becomes increasingly more difficult. Chocking when eating/drinking becomes more common. • Reflexes become abnormal.

	• Seizures are possible. • Muscles grow rigid. • Generally bedridden. • Spends more time asleep. • Require round-the-clock care

1.7.2 Diagnosis of Alzheimer's disease:

A doctor can diagnose most cases of Alzheimer's. However, nobody can be 100% sure until after death, when a microscopic examination of the brain detects plaques and tangles. There is no basic testing, such as a blood test, urine test, biopsy, or image scan for diagnosing Alzheimer's disease. A brain scan may help identify changes in the brain.

Lund-Manchester Criteria for Frontotemporal Dementia:

The Lund-Manchester diagnostic criteria for frontotemporal dementia require all of the following core components to be present. (Wang et. al., 1999)

1. Insidious onset and gradual progression,
2. Early decline in social interpersonal conduct,
3. Early impairment in regulation of personal conduct,
4. Early emotional blunting,
5. Early loss of insight.

Supportive diagnostic features include:

A. Behavioral disorder:

a. decline in personal hygiene and grooming
b. mental rigidity and inflexibility
c. distractibility and impersistence
d. hyperorality and dietary change
e. utilization behavior

B. Speech and language:

Altered speech output (aspontaneity and economy of speech, press of speech), stereotypy of speech, echolalia, perseveration, mutism.

C. Physical signs:

Primitive reflexes, incontinence, akinesia, rigidity, tremor, low/labile blood pressure.

D. Investigations:

a. Neuropsychology: impaired frontal lobe tests; no amnesia or perceptual deficits
b. EEG: normal on conventional EEG despite clinically-evident dementia
c. Brain imaging: predominant frontal and/or anterior temporal abnormality

1.8. Antioxidant

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols. (Helmut, 1997)

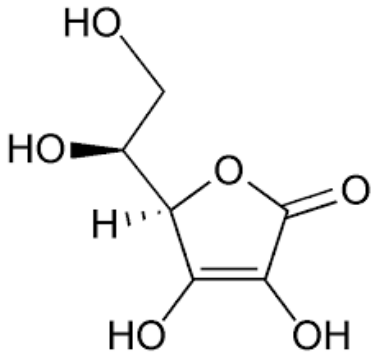

Fig 1: Chemical structure of Ascorbic acid (MediLexicon, 2016)

Substituted phenols and derivatives of phenylenediamine are common antioxidants used to inhibit gum formation in gasoline (petrol).

Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. Oxidative stress is damage to cell structure and cell function by overly reactive oxygen-containing molecules and chronic excessive inflammation. Oxidative stress seems to play a significant role in many human diseases, including cancers. The use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. For these reasons, oxidative stress can be considered to be both the cause and the consequence of some diseases.

Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness. Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials with a limited number of antioxidants detected no benefit and even suggested that excess supplementation with certain putative antioxidants may be harmful. (Bjelakovic et. al., 2007). Antioxidants also have many industrial uses, such as preservatives in food and cosmetics and to prevent the degradation of rubber and gasoline (Dabelsteine et. al., 2007)

1.8.1 Free radical

Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules. Once formed these highly reactive radicals can start a chain reaction, like dominoes. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA, or the cell membrane. Cells may function poorly or die if this occurs. To prevent free radical damage the body has a defense system of antioxidants.

Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Although there are several enzyme systems within the body that scavenge free radicals, the principle micronutrient (vitamin) antioxidants are vitamin E, beta-carotene, and vitamin C. Additionally, selenium, a trace metal that is required for proper function of one of the body's antioxidant enzyme systems, is sometimes included in this category. The body cannot manufacture these micronutrients so they must be supplied in the diet.

1.8.2 DPPH

DPPH is a common abbreviation for an organic chemical compound 2,2-diphenyl-1-picrylhydrazyl. It is a dark-colored crystalline powder composed of stable free-radical molecules. DPPH has two major applications, both in laboratory research: one is a monitor of chemical reactions involving radicals, most notably it is a common antioxidant assay and another is a standard of the position and intensity of electron paramagnetic resonance signals.

1.8.3 Types of Antioxidants

"For example, the role of vitamin C is to stop the chain reaction before it starts," he says. "It captures the free radical and neutralizes it. Vitamin E is a chain-breaking antioxidant. Wherever it is sitting in a membrane, it breaks the chain reaction."

Flavonoids are the biggest class of antioxidants. Researchers have identified some 5,000 flavonoids in various foods.

Polyphenols are a smaller class of antioxidants, which scientists often refer to as "phenols." (Terms like phytonutrient and phytochemical are more generic terms that researchers sometimes use to describe nutrients and chemicals in plants.)

"We have clear science about antioxidants, that our bodies need a Natural Antioxidant Defense Network, for lack of a better term," Blumberg says. "Just like a country needs a military system, the human body needs defense workers at all levels -- lieutenants, corporals, generals, staff sergeants - in the form of antioxidants."

1.8.4 Mechanism of antioxidant

Blueberries, pomegranates, green tea, and dark chocolate—these are just some of the antioxidant-rich "superfoods" found in almost any supermarket today. As well as improving our general health, there is growing evidence that diets high in antioxidants may confer some protection against a long list of chronic diseases, including Alzheimer's disease, cancer, and even HIV. Given their increasing popularity, the fundamental question bears asking: What exactly are antioxidants, and how do they work in our bodies?

Antioxidants come in several forms, including the vitamins A, C, and E; plant-derived polyphenols, found in colorful fruits and vegetables; and also the element selenium, found in nuts and broccoli. "What these compounds share," explains K. Sandeep Prabhu, Penn State assistant professor of immunology and molecular toxicology, "is the ability to neutralize harmful molecules in our cells."

These harmful molecules, known as free radicals, contain unpaired electrons—which is unusual because electrons typically come in pairs. "The unpaired electrons make free radicals highly reactive, and in this state, they can cause damage by attacking the components of our cells, and can even cause cancer," Prabhu says.

So where do free radicals come from? Some are created as a natural by-product of reactions in our cells, says Prabhu. Other sources of free radicals include cigarette smoke, air pollution, and exposure to UV light or radiation. And once free radicals are formed, they can make more free radicals by scavenging electrons from other molecules, "creating a domino effect," he adds.

Antioxidants neutralize free radicals either by providing the extra electron needed to make the pair, or by breaking down the free radical molecule to render it harmless. "Antioxidants stop the chain reaction of free radical formation and benefit our health by boosting our immune system," explains Prabhu. Because antioxidants are used up in the process of free radical neutralization, a diet rich in antioxidants is essential to ensure a constant supply.

Research has shown that antioxidants can have an important impact on serious diseases. In one recent study, the addition of a polyphenol-rich blueberry gel to the diet of oral cancer patients prevented recurrence of the cancer. Another experiment demonstrated that increased levels of selenium in the diets of a group of HIV-positive patients significantly delayed progression of the disease.

In light of these impressive results, should everyone be taking antioxidant diet supplements? Prabhu warns that there can be too much of a good thing: "As with most things, excessive levels of antioxidants can be toxic." Furthermore, he stresses, "We don't yet fully understand

the mechanisms by which selenium and other antioxidants work, and so we must be cautious about prescribing diets high in these elements." In the Prabhu Lab, work is currently underway to discover how selenium works, with the goal of introducing selenium as a therapy for HIV. (Prabhu, 1996)

A diet containing a balance of the various forms of antioxidants will maintain overall good health, and could even impact serious diseases. For instance, the American Cancer Society encourages people to eat five servings of fruits and vegetables per day, and emphasizes the benefits of getting your antioxidants through foods rather than supplements. Prabhu himself makes sure he gets the recommended daily allowance of selenium by eating a few brazil nuts every day. "The key," says Prabhu, " is to eat a variety of fruits, vegetables, and nuts to ensure that we are taking advantage of all the health benefits that antioxidants can provide."

Free radicals contain an unpaired electron. These radicals can have a negative effect on cells causing oxidative damage that leads to cell death. Antioxidants prevent cell damage by binding to the free radical and neutralizing its unpaired electron. For example, when vitamin E binds to OO^- or O_2^- they form an intermediate structure that is converted to Alpha-tocopherylquinone.

1.8.5 History of antioxidant

As part of their adaptation from marine life, terrestrial plants began producing non-marine antioxidants such as ascorbic acid (Vitamin C), polyphenols and tocopherols. The evolution of angiosperm plants between 50 and 200 million years ago resulted in the development of many antioxidant pigments – particularly during the Jurassic period – as chemical defences against reactive oxygen species that are byproducts of photosynthesis. (Benzie,2003). Originally, the term antioxidant specifically referred to a chemical that prevented the consumption of oxygen. In the late 19th and early 20th centuries, extensive study concentrated on the use of antioxidants in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines.(Mattill, 1947).

Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity. (German, 1999). Antioxidant activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption. However, it was the identification of vitamins A, C, and E as antioxidants that revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms. (Knight, 1998). The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with anti-oxidative activity is likely to be one that is itself readily oxidized. (Maureu et. al., 1922)Research into how vitamin E prevents the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevent oxidative reactions, often by scavengingreactive oxygen species before they can damage cells (George, 2005).

1.8.6 Metabolites of antioxidant

Some antioxidants are only found in a few organisms and these compounds can be important in pathogens and can be virulence factors (Miller, 1997)

The relative importance and interactions between these different antioxidants is a very complex question, with the various metabolites and enzyme systems having synergistic and interdependent effects on one another (Chaudiere, Ferrari-Illiou, 1999). The action of one antioxidant may therefore depend on the proper function of other members of the antioxidant system. The amount of protection provided by any one antioxidant will also depend on its concentration, its reactivity towards the particular reactive oxygen species being considered, and the status of the antioxidants with which it interacts (Vertuani et. al., 2004) .

Some compounds contribute to antioxidant defense by chelatingtransition metals and preventing them from catalyzing the production of free radicals in the cell. Particularly important is the ability to sequester iron, which is the function of iron-binding proteins such as transferrin and ferritin. (Ames et.al., 1981) Selenium and zinc are commonly referred to as antioxidant nutrients, but these chemical elements have no antioxidant action themselves and are instead required for the activity of some antioxidant enzymes, as is discussed below:-

Table 1.4: Antioxidant Metabolite

Antioxidant metabolite	**Solubility**	**Concentration in human serum (µM)**	**Concentration in liver tissue (µmol/kg)**
Ascorbic acid (vitamin C)	Water	50 – 60	260 (human)
Glutathione	Water	4	6,400 (human)
Lipoic acid	Water	0.1 – 0.7	4 – 5 (rat)
Uric acid	Water	200 – 400	1,600 (human)
Carotenes	Lipid	β-carotene: 0.5 – 1 retinol (vitamin A): 1 – 3	5 (human, total carotenoids)
α-Tocopherol (vitamin E)	Lipid	10 – 40	50 (human)
Ubiquinol(coenzyme Q)	Lipid	5	200 (human)

(Evelson et. al., 2001) (Morrison et. al., 1999)

1.8.7 Pro-oxidant activities

Antioxidants that are reducing agents can also act as pro-oxidants. For example, vitamin C has antioxidant activity when it reduces oxidizing substances such as hydrogen peroxide, (Halliwell B., 2008) however, it will also reduce metal ions that generate free radicals through the Fenton reaction. (Ristow, Zarse, 2010)

$$2\ Fe^{3+} + \text{Ascorbate} \rightarrow 2\ Fe^{2+} + \text{Dehydroascorbate}$$

$$2\ Fe^{2+} + 2\ H_2O_2 \rightarrow 2\ Fe^{3+} + 2\ OH\cdot + 2\ OH^-$$

The relative importance of the antioxidant and pro-oxidant activities of antioxidants are an area of current research, but vitamin C, which exerts its effects as a vitamin by oxidizing polypeptides, appears to have a mostly antioxidant action in the human body. (Magnenat et. al., 1998) However, less data is available for other dietary antioxidants, such as vitamin E, or the polyphenols. (Stanner et. al., 2004) Likewise, the pathogenesis of diseases involving hyperuricemia likely involve uric acid's direct and indirect pro-oxidant properties.

That is, paradoxically, agents which are normally considered antioxidants can act as conditional pro-oxidants and actually increase oxidative stress. Besides ascorbate, medically important conditional pro-oxidants include uric acid and sulfhydryl amino acids such as homocysteine. Typically, this involves some transition-series metal such as copper or iron as catalyst. The potential role of the pro-oxidant role of uric acid in (e.g.) atherosclerosis and ischemic stroke is considered above. Another example is the postulated role of homocysteine in atherosclerosis.

1.8.8 Enzyme systems of antioxidant

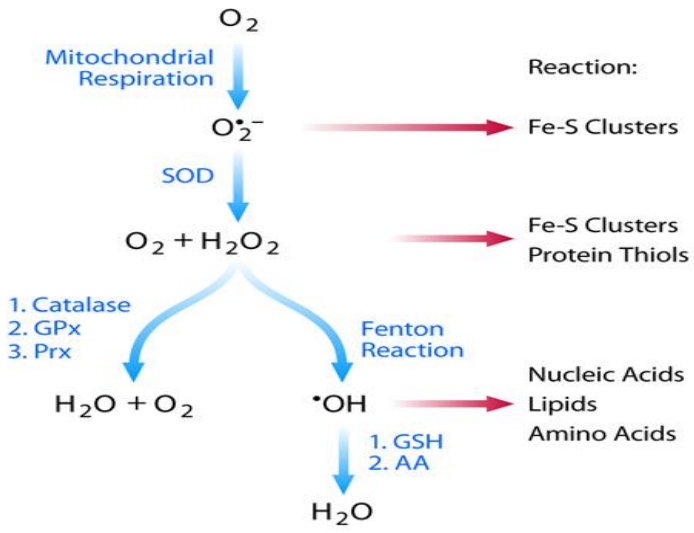

Fig 2: Enzymatic pathway for detoxification of reactive oxygen species. (Impact journal, 2016)

As with the chemical antioxidants, cells are protected against oxidative stress by an interacting network of antioxidant enzymes. (Herberg et. al., 2004). Here, the superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is the result of multiple enzymes, with superoxide dismutases catalysing the first step and then catalases and various peroxidases removing hydrogen peroxide. As with antioxidant metabolites, the contributions of these enzymes to antioxidant defenses can be hard to separate from one another, but the generation of transgenic mice lacking just one antioxidant enzyme can be informative. (Hurrell, 2003)

1.8.9 Adverse effects of antioxidant

Relatively strong reducing acids can have antinutrietive effects by binding to dietary minerals such as iron and zinc in the gastrointestinal tract and preventing them from being absorbed. (Beecher, 2003). Notable examples are oxalic acid, tannins and phytic acid, which are high in plant-based diets. (Prashar et. al., 2006). Calcium and iron deficiencies are not uncommon in diets in developing countries where less meat is eaten and there is high consumption of phytic acid from beans and unleavened whole grain bread. (Hornig et. al., 1980)

Table 1.5 : Reducing acid present in Foods

Foods	Reducing acid present
Cocoa bean and chocolate, spinach, turnip and rhubarb.	Oxalic acid
Whole grains, maize, legumes.	Phytic acid
Tea, beans, cabbage.	Tannins

(Valko et. al., 2005)

Nonpolar antioxidants such as eugenol—a major component of oil of cloves—have toxicity limits that can be exceeded with the misuse of undiluted essential oils. Toxicity associated with high doses of water-soluble antioxidants such as ascorbic acid are less of a concern, as these compounds can be excreted rapidly in urine (Dabies, 1995). More seriously, very high doses of some antioxidants may have harmful long-term effects. The beta-Carotene and Retinol Efficacy Trial (CARET) study of lung cancer patients found that smokers given supplements containing beta-carotene and vitamin A had increased rates of lung cancer. Subsequent studies confirmed these adverse effects. (Bjelakovic, et. al., 2007)

These harmful effects may also be seen in non-smokers, as a recent meta-analysis including data from approximately 230,000 patients showed that β-carotene, vitamin A or vitamin E supplementation is associated with increased mortality but saw no significant effect from vitamin C. (Hurrel, 2003). No health risk was seen when all the randomized controlled studies were examined together, but an increase in mortality was detected only when the high-quality and low-bias risk trials were examined separately. As the majority of these low-

bias trials dealt with either elderly people, or people already suffering disease, these results may not apply to the general population. (Bjelakovic et. al., 2006). this meta-analysis was later repeated and extended by the same authors, with the new analysis published by the Cochrane Collaboration; confirming the previous results (Coulter et. al., 2006). These two publications are consistent with some previous meta-analyzes that also suggested that Vitamin E supplementation increased mortality, (Schumacker, 2006) and that antioxidant supplements increased the risk of colon cancer. (Seifried et. al., 2003) However, the results of this meta-analysis are inconsistent with other studies such as the SU.VI.MAX trial, which suggested that antioxidants have no effect on cause-all mortality (Block et. al., 2007). Overall, the large number of clinical trials carried out on antioxidant supplements suggest that either these products have no effect on health, or that they cause a small increase in mortality in elderly or vulnerable populations. (Gibson et. al., 2006)

While antioxidant supplementation is widely used in attempts to prevent the development of cancer, antioxidants may interfere with cancer treatments, (Boozer et. al., 1955) since the environment of cancer cells causes high levels of oxidative stress, making these cells more susceptible to the further oxidative stress induced by treatments. As a result, by reducing the redox stress in cancer cells, antioxidant supplements could decrease the effectiveness of radiotherapy and chemotherapy. (Ceresana, 2016). On the other hand, other reviews have suggested that antioxidants could reduce side effects or increase survival times. (Innospec Chemicals, 2006)

1.9. Treatment Strategies of AD:

There is currently no cure for AD, however there are multiple drugs that have been proven to slow disease progression and treat symptoms. When initiating treatment for AD patients, physicians divide the symptoms into “cognitive” and “behavioral and psychiatric” categories. The treatment strategies are given below:

1.9.1. Cholinesterase inhibitors:

- Prevent the breakdown of acetylcholine, a chemical messenger important for learning and memory. This supports communication among nerve cells by keeping acetylcholine levels high.
- Delay worsening of symptoms for 6 to 12 months, on average, for about half the people who take them.
- Are generally well tolerated. If side effects occur, they commonly include nausea, vomiting, loss of appetite and increased frequency of bowel movements.

The cholinesterase inhibitors are commonly prescribed:

a. Donepezil (Aricept) is approved to treat all stages of Alzheimer's.
b. Rivastigmine (Exelon) is approved to treat mild to moderate Alzheimer's.

c. Galantamine (Razadyne) is approved to treat mild to moderate Alzheimer's.
d. Tacrine (Cognex) was the first cholinesterase inhibitor approved.

There are currently three cholinesterase inhibitors commonly prescribed: tacrine, donepezil, galantamine, and rivastigmine.

a. **Donepezil:**

Donepezil is a piperidine derivative that reversibly inhibits acetylcholinesterase. (Cori, Feri, 1999). It is often regarded as providing only symptomatic relief without providing neuroprotective effects. However, in vitro studies show that donepezil offers neuroprotection by reducing glutamate excitotoxicity, diminishing βA toxicity and consequently increasing cell longevity. It slowed atrophy of the hippocampus in humans, which suggest a neuroprotective effect. It is very well absorbed after oral administration and reaches peak plasmatic concentration (C_{max}) in 3-4 hours. Elimination half-life of donepezil is approximately 70 hours allowing once daily administration. It binds to plasma proteins in a proportion of 96% and is metabolized by isoenzyme 2D6 and 3A4 of cytochrome P450. Starting and minimal effective dose is 5 mg once daily. Maximal recommended dose is 10 mg daily. Overall, both the doses of 5 mg and 10 mg were beneficial, with the higher dose being marginally more effective. More side effects were reported with donepezil than with placebo. Most common side-effects were nausea, vomiting, diarrhoea, muscle cramps, dizziness, fatigue and anorexia and they were dose-dependent. (Schneider, 2005)

b. **Galantamine:**

Galantamine is a tertiary alkaloid agent that reversibly inhibits AChE. Galantamine, a natural AChEI (originally derived from the common snowdrop and other plants, but now synthesized), protects neurons and reduced cell death by modulating nicotinic receptors, which are significantly reduced in AD brains. In an animal model, galantamine also increased dopaminergic neurotransmission in the hippocampus, a brain area particularly important in memory. (Halliwell, 2008)

Galantamine is rapidly absorbed after oral administration and reaches C_{max} in approximately 1 hour. Elimination half-life is between 7 to 8 hours. It binds to plasma proteins in a proportion of 18% and is metabolized by isoenzyme 2D6 and 3A4 of cytochrome P_{450}. Galantamine is commercialized as an extended-release formulation allowing once-daily dosing. Starting dose of galantamine ER is 8 mg once daily. Minimal effective dose is 16 mg daily and maximal dose is 24 mg daily. Galantamine’s side effects are comparable to other ChEI’s and consist mainly of cholinergic gastrointestinal symptoms. (Davies, 1995)

c. **Rivastigmine:**

Rivastigmine is a carbamate derivative that reversibly inhibits both acetyl- (AChE) and butyryl- (BuChE) cholinesterase. It is the only ChEI with significant inhibition of BuChE. Butyrylcholinesterase is widely distributed in the central nervous system and may play a role

in cholinergic function and neurodegeneration. It is unclear how specific BuChE inhibition relates to rivastigmine's clinical effect. (Bjelakovic et. al., 2007)

Rivastigmine is well absorbed after oral administration and reaches C_{max} in one hour. Its elimination half-life is approximately 1 to 2 hours. It binds to proteins in a proportion of 40%, is hydrolysed by esterases (including cholinesterases) and is excreted in the urine. Cytochrome P_{450} isoenzymes are not involved in the metabolism of rivastigmine hence minimizing drug-drug interactions. Starting dose of rivastigmine is 1.5 mg twice a day and can be gradually titrated to the maximal dose of 6 mg twice a day. The minimal effective dose is 3mg twice a day. A transdermal form of rivastigmine has been developed and is available on most markets since 2008. (Stanner et. al., 2004). The main objective of transdermal rivastigmine is to allow titration to the highest (and most therapeutic) doses of the medication while minimizing side effects. This is achieved by slow release of the medication into the circulation as demonstrated by a C_{max} of 8 hours by transdermal route. Starting dose of transdermal rivastigmine is 5 cm^2 and the effective and maximal dose is 10 cm^2. More side-effects were reported with rivastigmine than with placebo and they were dose-dependent. Most common side effects were nausea, vomiting, diarrhoea, anorexia, headache, syncope, abdominal pain and dizziness.

d. **NMDA antagonist (Memantine):**

Memantine is thought to reduce cell damage by reducing excitotoxicity resulting from overactivation of NMDA glutamate receptor during synaptic transmission. (Shenkin, 2006).

In addition to cholinesterase inhibitors, memantine has also been approved for the treatment of AD. Memantine regulates the activity of glutamate in the brain. Glutamate is an excitatory neurotransmitter involved in learning and memory. Over stimulation of nerves by glutamate may be the cause of the neuron degeneration seen in AD, called Bethune 16 excitotoxicity. Glutamate binds to N-methyl-D-aspartate (NMDA) receptors on the surface of brain cells. Memantine functions by blocking the NMDA receptors and therefore protecting the nerves from excessive glutamate stimulation. (Hercberg et. al., 2004).

Memantine is indicated in the treatment of moderate to severe AD and can temporarily delay worsening of cognitive symptoms. It is well absorbed after oral administration and reaches C_{max} in 3 to 8 hours. Elimination half-life is 60-80 hours. It binds to proteins in a proportion of 45% and is almost completely excreted unchanged in the urine. Starting dose is 5 mg daily (in one or two doses). Minimal therapeutic dose is 10 mg daily and maximal dose is 20 mg daily. NMDA antagonists, such as memantine, have generally been regarded as neuroprotective, but they have also demonstrated neurotoxic properties that diminish memory, incite neuron death and even produce psychotic episodes in humans. (Hurrel, 2003)

1.9.2 Secretase inhibitors:

Secretase are the enzymes that break the APP, found in cell membranes, into βA fragments that form plaques. Secretase inhibitors should slow the production of βA. A gamma-secretase inhibitor has been shown to reduce plasma βA about 60 percentin a small 14 week study of mild to moderate AD patients. B-secretase inhibitors have been shown to reduce βA in animal models and may have fewer adverse effects. Memoquin is a β-secretase inhibitor that also inhibits AChE, reduces βA production, limits tau hyperphosphorylation and fights oxidation, but it is in the early developmental stage.

1.9.3. Insulin:

Insulin has many roles in normal cell function. Nasal administration of insulin improved several cognitive measures in subjects with early AD or mild cognitive impairment. Insulin resistance can affect the brain as well as other organs, making it difficult for the brain cells to acquire energy for cell maintenance and synaptic connections, thus cell death can occur. Also hyperinsulinemia has been found to increase inflammation and βA_{1-42} in healthy adults.

1.9.4. Etanercept:

Etanercept has recently generated interest because it produced dramatic cognitive improvement. AD brain have elevated lavels of the cytokines TNF-α. Since TNF-α regulates neural transmission, lowering by spinal injections of etenercept might restore the brain to more normal functioning. A dramatic cognitive imprvement was evedenced in one moderate to severe AD subject within minutes.

1.9.5. Brain Derived Neurotrophic factor (BDNF):

BDNF is a protein produced in the brain that help existing neuron survive, facilitate the growth of new neuron and synaps and reverses neuronal atrophy and behavior deficits; intracellular signalling is also facilitated. BDNF is active in the hippocampusand cortex. It stimulates neurogemesis. BDNF level decline with age and are lower in the AD brains than in whose without AD.

1.9.6. Immunization:

βA has been reduced by injecting AD patients with a synthetic form of βZ called AN1792. Although this reduces βA, the effect on AD is unclear. Some people respond to immunization without a showing of disease progression even after 4.6 years.

1.9.7.Antipsychotics and sedatives:

Antipsycotics and sedatives have accelarated the progression of AD, defines as an increase of one or more points in the Global deterioration Scale, and produced a 50% decrease in cortical plasticity in cats. Thus care should be exercrised in using such drugs for AD patients.

1.9.8 Flavanoids and other novel plant constituents:

a. HuperzineA (HupA):

HupA is an extract from the chinese moss Huperzia serrata that has been used for centuries in Chinese folk medicine to treat wide range of diseases.

A review of in vitro studies found HupA preserves Ach longer than Tacrine, Galantamine or Donepezil. HupA reduces βA induced neuronal degenaration in the hippocampus and the cortex, decrease oxidative damage from free radical induced βA plaque, protects neuron from cytotoxins and apoptosis induced by βA and free radicals and inhibit glutamate toxicity.

b. Polyphenols:

Polyphenols are group of plant derived chemical compunds with more than one phenol units. They protect the plant from stress induced by ultraviolet radiation, disease, pests and physical damage. Polyphenols also protect animals by activating a number of intracellular processes that preserve neurons.

c. Curcumin:

Curcumin is extracted from the plant Curcuma longa (turmeric). It may provide promising therapy for AD because it has at least 10 neuroprotective properties including anti-inflammatory, antioxidant, inhibition of βA formation, clearance of βA formation and copper and iron chelation.

d. Reveratrol:

Reveratrol is a polyphenol found in red wine, peanuts and other plants which reduce oxidative stress, decrease inflammation, reduce βA formation, protect DNA, decrease cell death and modulates various other systemsthat protect cells. Animal models suggest that reveratrol mimics the effect of caloric restriction on longivity and negates the harmful effects of high fat diet, doubles resestence to muscle fatigue, reduce neurotoxicity, decrease cell death, decrease degeneration of the hippocampus and prevents learning impairments. Several studies has shown thatmoderate consumption of red wine reduces the risk of developing AD.

e. Tacrine(Cognex):

Tacrine was the first cholinesterase inhibitor approved. Doctors rarely prescribe it today because it's associated with more serious side effects than the other three drugs in this class.

1.9.9. Herbal suplement:

a. Gingko Biloba:

Gingko Biloba contains compounds that have antioxidant and antiinflammatory properties that protects neuron membrane, regulate neurotransmitters and retard cell degeneration. Gingko Biloba extract contains RGb 761that reduce βA and cell death.

b. Panax ginseng:

Panax ginseng has been studied for its effect of cognition. The active componant in ginseng are thought to be steroid like compounds called ginsenosides. Ginsenosides Rg3 reduced βA_{1-42} by 84% invitro and 31% invivo.

c. Withania sominifera:

Withania sominifera, a small evergreen hrub commonly called ashwagandha has been used to treat many diseases. A recent study have been shown that it have many neuroprotective properties including anti-inflammatory, antioxidant, inhibition of βA, inhibition of Calcium, inhibition of AChE and reduction of cell death. In vitro research demonstrates that ir regenate damaged axons, dendrites and synaps.

1.9.10 Nutrients:

a. Posphatidylserine:

Posphatidylserine is important in neurotransmission, mitochondrial function and cell metabolism. It has also been implecated in the enhancement of nerve growth factor. Recent research demonstrate that Posphatidylserine increases Ach and provides neuroprotection by inhibiting βA and inflammation.

b. Alpha-Lipoic acid (ALA):

ALA is a fatty acid found in all cells and in some food. It is a powerfull antioxidant that readily penetrates the blood brain barrie, chelate metals, reduce inflammaiton and increase ACh.

c. Omega-3 Fatty Acids:

Omega-3 Fatty Acids have many benificial effectsthat make them investigative prospects of AD. Daily taking of Omega-3 Fatty Acids reduces the risks of developing AD. It has also benificial effects on mild to moderate AD.

d. Acetyl L-Carnitine (ALCAR):

ALCAR derived from amino acid L-carnitine, work synergestically with ALA to transport acyl groups and fatty acids into the mitochondria for energy produciton. IT is a small molecule that readily penetrates the BBB and promotes biosynthesis of Ach. It also clears the toxic fatty acid metabolites. ALCAR also increases nerve growth factors.

e. Coenzyme Q10:

Coenzyme Q10 is essential for mitochondrial energy production. Many mitochondrial dysfunction occurs in AD brains, including disruption of energy production, apoptosis deregulation, altered calcium homeostasis and others. Coenzyme Q10 reduced oxidative stress and tau pathology in mice and metabolized βA and inhibited its formation invitro.

1.10. Plants as a source of AD drugs:

None of the pharmacological lines of intervention have so far been able to stop the progression of AD; thus a need for an alternative approach was believed necessary to make progress with particular emphasis on plants. Plants have been used since antiquity in traditional medicinal systems for the treatment of memory dysfunction. Studies carried out on some species have resulted in the identification of compounds which are currently either in clinical use or templates for further drug discovery, e.g. galantamine, an alkaloid isolated from *Galanthusnivalis* L. (Amaryllidaceae). Galantamine was approved by the FDA in 2004 for use an acetylcholinesterae inhibitor in the treatment of AD. It was the traditional use of of *G. nivalis* L. in Bulgaria and Turkey for neurological conditions that lead to the development of this drug. (Albenes, 1999)

There have been previous reviews on the plants demonstrating pharmacological and clinical effects of potential interest in AD therapy, including (Clement et al., 2003), (Howes and Houghton, 2003), (Howes et. al., 2003) and (Zhang, 2006). By studying the reviews it becomes clear that ethopharmacological screening is one of the main approaches used in drug discovery. Some of the preclinical and clinical studies related to AD, carried out with medicinal plant have been mentioned in Table 1.4. The following is a review of the plants and the phytochemical substances which have shown to be of therapeutic potential in AD.

1.11. Cholinesterase and their mechanism:

1.11.1. Acetylcholinesterase:

Acetylcholinesterase (AChE) is key enzyme in the nervous system of animals. It is ahydrolasethathydrolyzesthe neurotransmitter acetylcholine. AChE is found at mainly neuromuscular junctions and cholinergic brain synapses, where its activity serves to terminate synaptic transmission. (Benthal, 1933). It belongs to carboxylesterase family of enzymes. It is the primary target of inhibition by organophosphorus compounds such as nerve agents and pesticides.

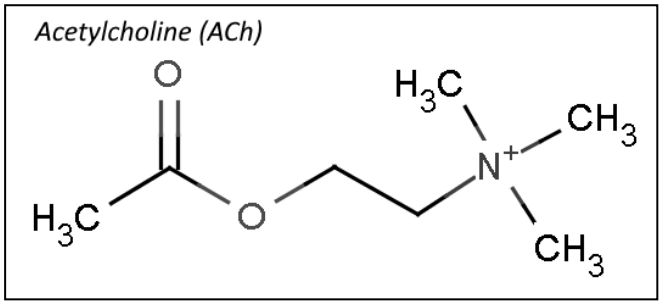

Fig 3: Chemical Structure of acetylcholine. (PsychiatricDrugs, 2016)

1.11.1.1. Enzyme structure and mechanism:

AChE has a very high catalytic activity - each molecule of AChE degrades about 25000 molecules of acetylcholine (ACh) per second, approaching the limit allowed by diffusion of the substrate. (Brown et. al., 1954). The active site of AChE comprises 2 subsites - the anionic site and the esteraticsubsite. The structure and mechanism of action of AChE have been elucidated from the crystal structure of the enzyme.

The anionic subsite accommodates the positive quaternary amine of acetylcholine as well as other cationic substrates and inhibitor. The cationic substrates are not bound by a negatively-charged amino acid in the anionic site, but by interaction of 14aromaticresidues that line the gorge leading to the active site. All 14 amino acids in the aromatic gorge are highly conserved across different species. Among the aromatic amino acids, tryptophan 84 is critical and its substitution with alanineresults in a 3000-fold decrease in reactivity. (Chaturvedi, 1985). The gorge penetrates half way through the enzyme and is approximately 20 angstroms long. The active site is located 4 angstroms from the bottom of the molecule. (Irvine, 1961)

The esteraticsubsite, where acetylcholine is hydrolyzed to acetate and choline, contains the catalytic triad of three amino acids: serine 200, histidine 440 and glutamate 327. These three amino acids are similar to the triad in other serine proteases except that the glutamate is the third member rather than asparate. Moreover, the triad is of opposite chirality to that of other proteases. (Roy, 1987) The hydrolysis reaction of the carboxyl ester leads to the formation of an acyl-enzyme and free choline. Then, the acyl-enzyme undergoes nucleophilic attack by a water molecule, assisted by the histidine 440 group, liberating acetic acid and regenerating the free enzyme. (Jena, 1991)

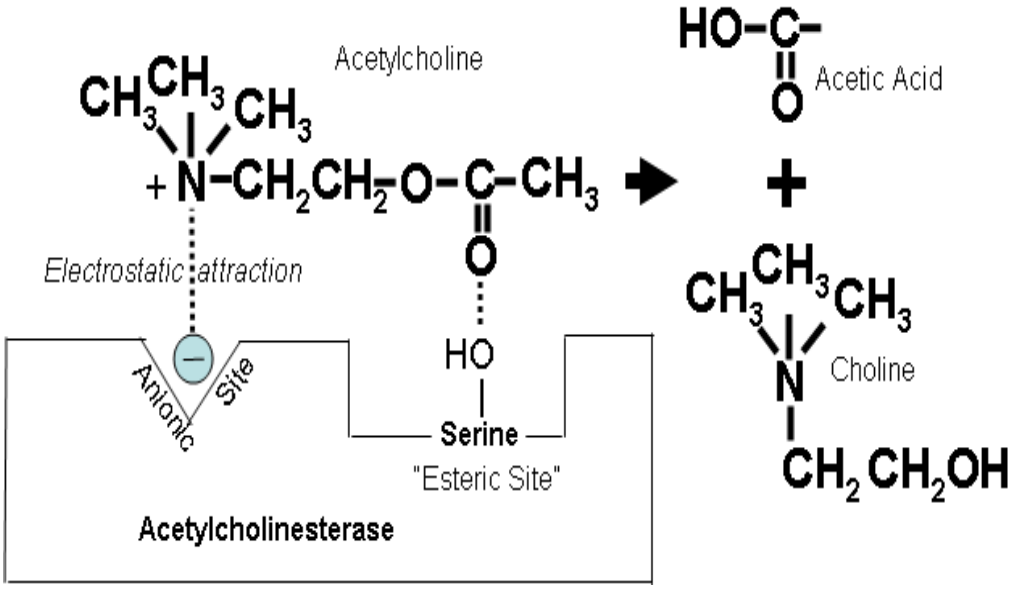

Fig. 4: Mechanism of action of AChE. (ATSDR, 2016)

1.11.1.2. Distribution of AChE:

AChE is found in many types of conducting tissue: nerve and muscle, central and peripheral tissues, motor and sensory fibers, and cholinergic and noncholinergic fibers. The activity of AChE is higher in motor neurons than in sensory neurons. (Storrs, 1995)

Acetylcholinesterase is also found on the red blood cell membranes, where different forms constitute the Yt blood group antigens. Acetylcholinesterase exists in multiple molecular forms, which possess similar catalytic properties, but differ in their oligomeric assembly and mode of attachment to the cell surface.

1.11.1.3. Biological function of AChE:

During neurotransmission, ACh is released from the nerve into the synaptic cleft and binds to ACh receptors on the post-synaptic membrane, relaying the signal from the nerve. AChE, also located on the post-synaptic membrane, terminates the signal transmission by hydrolyzing ACh. The liberated choline is taken up again by the pre-synaptic nerve and ACh is synthetized by combining with acetyl-CoA through the action of choline acetyltransferase. (Storrs., 1995)

1.11.2. Butyrylcholinesterase enzyme:

Butyrylcholinesteraseis a non-specific cholinesterase enzyme that hydrolyses many different choline esters. It is also known as pseudocholinesteras or plasma cholinesterase. In humans, it is found primarily in the liverand is encoded by the *BCHE* gene. (Mathur, 2001)

It is very similar to the neuronal acetylcholinesterase, which is also known as RBC or erythrocyte cholinesterase.The term "serum cholinesterase" is generally used in reference to a clinical test that reflects levels of both of these enzymes in the blood. (Reddy et. al., 1979)

Assay of butyrylcholinesterase activity in plasma can be used as a liver function test as both hypercholinesterasemia and hypocholinesterasemia indicate pathological processes. (Reddy et. al., 1979)

1.12. Enzyme Kinetics:

Enzyme kinetics is the study of the chemical reactions that are catalyzed by enzyme. In enzyme kinetics, the reaction rate is measured and the effects of varying the conditions of the reaction are investigated. Studying an enzyme's kinetics in this way can reveal the catalytic mechanism of this enzyme, its role in metabolism, how its activity is controlled, and how a drug or an agonist might inhibit the enzyme.

Enzymes are usually protein molecules that manipulate other molecules, like the enzymes substrates. These target molecules bind to an enzyme's active site and are transformed intoproducts through a series of steps known as the enzymatic mechanism. These mechanisms can be divided into single-substrate and multiple-substrate mechanisms. Kinetic studies on enzymes that only bind one substrate, such as triosephosphateisomerase, aim to measure the affinitywith which the enzyme binds this substrate and the turnover rate. Some

other examples of enzymes are phosphofructokinase and hexokinase, both of which are important for cellular respiration (glycolysis). (Khoja, Halbe, 2001)

1.13. Objectives:

Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting the brain. It is the most common cause of dementia, leading to deterioration in vital cognitive process such as memory, understanding and speech. About 25 million people are estimated to suffer from this disease all over the world and 66% of them live in the third world countries. These data demand for an effective treatment of AD.

AD is a multifactorial disease. Although several factors have been identified in the etiology and pathogenesis of AD, cholinergic dysfunction and oxidative stress have been implicated as the major contributing factors in the pathogenesis of AD. Therefore inhibition of acetylcholinesterase and oxidation are the two promising strategies for the development of drug in the treatment of AD.

The loss of cholinergic function in the central nervous system is an important feature of AD, which is accompanied by deficiency of acetylcholine (ACh). The level of acetylcholine in AD correlates well with the cognitive decline and severity of the disease. Inhibition of acetylcholinesterase, which enhances cholinergic transmission by reducing the enzymatic degradation of acetylcholine, is thus an important approach for the development of AD drug. Acetylcholiesterase inhibitors are the first group of drugs currently approved by FDA to treat of AD. Currently, five drugs are approved by the Food and Drug Administration (FDA) for the treatment of AD, four of them are acetylcholinesterase inhibitors (donepezil, galantamine, rivastigmine, and tacrine). These medications ameliorate the symptoms and can improve the functioning of patients with AD, but they are not curative, nor do they significantly change the course of the illness. Moreover, these approved drugs are limited in use due to their adverse side effects such as gastrointestinal disturbance and bioavailability problems.

Oxidative stress is another important feature of AD. Excessive lipid peroxidation, protein oxidation, DNA and RNA oxidation, glycoxidation have all been documented in AD brains. Therefore, antioxidant therapies envisaging the reduction of oxidative damage and the increase of endogenous antioxidant defenses have been suggested to prevent, delay, or ameliorate the disease symptoms. At present there is special interest on natural antioxidants derived from the plant resources which are candidates in the prevention of oxidative damage.

*T. indica*barks (locally called Tetul) belging to the indica family has been used for centuries in folk medicine for the treatment of center nervous system (CNS) disorders including AD. The plant is traditionally used in the treatment of women's diseases particularly in menstrual trouble, chronic wound, diarrhoea and skin infection.

Previous reports from our laboratory have shown that the extracts of *T. indica* possess strong antimicrobial activity and contain important bioactive compounds including catechin, 3,4-dihydroxy-cinnamyl alcohol and 2,3,4- trihydroxy-cinnamyl alcohol. These compounds have

been demonstrated earlier to have antioxidant and neuroprotective properties and thus may play a role in the anti-inflammatory activity. Although *L. globosus*has important medicinal values to be used in the treatment of AD, no works have been carried out yet for its anti-AD activity. Therefore the present work is designed to evaluate the neuroprotective and antioxidant activities of the extract of *L. globosus*. Beside this determination of the kinetics of AChE and BuChE enzyme inhibition and type of the inhibition is also in our concern.

1.14. Present Study Protocol:

The present study insights into the phytochemical and biological investigations includes:

- Extraction of the dried powder of barks with methanol by cold extraction method.
- Determination of total phenolic and flavonoid content of the methanol extract and its different fractions by Folin–Ciocalteu reagent and aluminium chloride respectively.
- *In vitro* assessment of different fractions for antioxidant activity by DPPH radical scavenging assay and for anti-cholinesterase activity by modified Ellman coupled enzyme assay.
- Isolation and purification AChE enzyme from bovine and rat brain source.
- Isolation and purification BuChE enzyme from human blood plasma.
- *In vitro* assessment of the isolated pure compounds for acetylcholinesterase inhibitory activity by modified Ellman method.
- *In vitro* assessment of isolated pure compounds for butyrylcholinesterase inhibitory activity by modified Ellman method.

Chapter 2

Literature Survey And Plant Profile

LITERATURE SURVEY AND PLANT PROFILE

2.1.Botanical Name: Tamarindus indica L.

Synonym: Tamarindus occidentalis, Tamarindus officinalis , Tamarindus umbrosa Salisb.

Common Name: Tamarind

English Name: Tamarind Tree.

Bengali/Vernacular Name: Tetul, Ambli.

Scientific Name: *Tamarindus indica* L.

(The Plant List, 2004)

2.2. Scientific Classification

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Fabales

Family: Fabaceae

Genus: Tamarindus L.

Species: *Tamarindus indica* L.

2.3. Geographical Distribution

Tamarind is native to tropical Africa, where it grows wild throughout the Sudan. It has been so long ago introduced into India that it has often been reported as indigenous there also. (Morton, 1987)

2.4. Plant morphology:

The tamarind is a long-lived, medium-growth, bush, which attains a maximum crown height of 12 to 18 metres (39 to 59 ft). The crown has an irregular, vase-shaped outline of dense foliage.

The tree grows well in full sun in clay, loam, sandy, and acidic soil types, with a high resistance to drought and aerosol salt (wind-borne salt as found in coastal areas).

The evergreen leaves are alternately arranged and pinnately compound. The leaflets are bright green, elliptical ovular, pinnately veined, and less than 5 cm (2.0 in) in length. The branches droop from a single, central trunk as the tree matures and is often pruned in agriculture to optimize tree density and ease of fruit harvest. At night, the leaflets close up.

The tamarind does flower, though inconspicuously, with red and yellow elongated flowers. Flowers are 2.5 cm wide (one inch), five-petalled, borne in small racemes, and yellow with orange or red streaks. Buds are pink as the four sepals are pink and are lost when the flower blooms. (Doughari, 2006)

The fruit is an indehiscentlegume, sometimes called a pod, 12 to 15 cm (4.7 to 5.9 in) in length, with a hard, brown shell.

Tamarind seedling (Walker magnum, 2012)

Tamarind flowers (DPREVIEW, 2016)

Tamarind leaves (Wikia, 2016)

Tamarind fruits (Organic Facts, 2016)

Fig 5: Different parts of *Tamarindus indica* plant.

2.5. Cultivation:

Seeds can be scarified or briefly boiled to enhance germination. They retain germination capability after several months if kept dry.

The tamarind has also long been naturalized in Indonesia, Malaysia, the Philippines, and the Pacific Islands. Thailand has the largest plantations of the ASEAN nations, followed by Indonesia, Myanmar, and the Philippines. The pulp is marketed in northern Malaya. It is cultivated all over India, especially in the South Indian states of Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu. Extensive tamarind orchards in India produce 275,500 tons (250,000 metric tons) annually. Commercial plantations throughout tropical Latin America include Brazil, Costa Rica, Colombia, Cuba, Guatemala, Mexico, Nicaragua, Puerto Rico and Venezuela.

In the United States, it is a large-scale crop introduced for commercial use, second in net production quantity to India, in the mainly Southern states due to tropical and semitropical climes, notably South Florida, and as a shade and fruit tree, along roadsides and in dooryards and parks.

2.6. Uses of various parts of *T. indica*

2.6.1. Fruit pulp :

Tamarind is valued mostly for its fruit, especially the pulp, which is used for a wide variety of domestic and industrial purposes. The acidic pulp is used as a favorite ingredient in culinary preparations, such as curries, chutneys, sauces, ice cream, and sherbet in countries where the tree grows naturally, In India, the pulp is also eaten raw and sweetened with sugar. Tamarind pulp is also used to make sweet meats mixed with sugar called Tamarind balls. (Letsohert, Beese, 1994). Tamarind pulp is used as a raw material for the manufacture of several industrial products, such as Tamarind Juice Concentrate, Tamarind Pulp Powder, tartaric acid, pectin, tartarates, and alcohol. (Purseglove, 1987).

2.6.2. Seed :

Tamarind seed is a by-product of the commercial utilization of the fruit, the seed comprises the seed coat or testa (20-30%) and the kernel or endosperm (70-75%). However, it has several uses. It is commercially available as a food additive for improving the viscosity and texture of processed foods. (Coronel, 1991). The name “jellose” has been suggested for the seed polysaccharide as it describes both its jelly forming properties and the carbohydrate character.

(Rao, 1948). It has been recommended for use as a stabilizer in ice-cream, mayonnaise, and cheese and as an ingredient or agent in a number of pharmaceutical products, and the seed oil is said to be palatable and of culinary quality. (Sone, Santo, 1994). The oil is used for making varnish to paint idolsand light lamp. (Morton, Miami, 1987)

2.6.3. Flowers and leaves :

The leaves, flowers, and immature pods of Tamarind are also edible. The leaves and flowers are used to make curries, salads, stews, and soups in many countries, especially in times of scarcity. These are used in some Thai food recipes because of their sourness and specific aroma. Children in Gambia mix the acid leaves with gum from fig trees to make a chewing gum. (Lewis, Nilakantan, 1964). The leaves and flowers are also useful as a mordant in dyeing. A yellow dye derived from the leaves colors wool red and turns indigo dyed silk to green. Mature leaves are used as a bleaching agent in the preparation of young leaves of "buri" (Corypha alata) for hat making in the Philippines. (Sozolnoki, 1985)

2.6.4. Wood :

Tamarind wood has many uses, including making furniture, wheels, mallets, rice pounders, mortars, pestles, ploughs, well construction, tent pegs, canoes, side planks for boats, cart shafts and axles, and naves of wheels, toys, oil presses, sugar presses, printing blocks, tools and tool handles, turnery, and so on. (Brown, 1954). In North America, Tamarind wood has been traded under the name of "Madeira mahogany" It is valued for making gunpowder. (Irvine, 1961) The ash is used to remove hair from animal hides and can be mixed with fruit pulp for cleansing and brightening brass and copper vessels. (Kulkarni et. al., 1993)

2.6.5. Seed testa and bark :

The seed testa contains 23% tannin, in leather tanning tests, Tamarind tannin gives harsh and highly colored leather, which could be used for heavy soles, suitcases, and others. The seed husk has also been found to be an effective fish poison. Bark tannins are used in the preparation of ink and for fixing dyes. (Jena, 1991)

2.6.6. Tamarind kernel powder :

Tamarind Kernel Powder (TKP) produced from the seeds is another commercial product and is often reported in commercial digests. (Storrs, 1995) The TKP will become rancid and brown if stored inadequately and the storage ability and color will be better if it is defatted. In India, TKP is used as a source of carbohydrate as the adhesive or binding agent in paper and textile sizing, and weaving and making jute products as well as textile printing. (Reddy et. al., 1979)

2.7. Chemical Constituents:

Thirty two fatty acids, two other compounds 9 beta, 19-Cyclo-4 beta 4, 4, 14, x-trimethyl-5 alpha-cholestan-3 beta-ol, 24R-Ethyl cholest-5-en, 3 beta-ol and 12 essential elements viz., Arsenic, Calcium, Cadmium, Copper, Iron, Sodium, Manganese, Magnesium, Potassium, Phosphorus, Lead, and Zinc were isolated from Tamarindus indica medicinal Plant. Accumulation of Copper was the lowest in T. indica while Potassium present with highest accumulation. Total protein in T. indicia was 7.5 to 6.6 %. (Khoja, Halbe, 2001)

2.8. Food Uses:

Tamarind is used as food in various ways. The tender, immature, very sour pods are cooked as seasoning with rice, fish and meats in India.

In Bahamas, the fully-grown, but still unripe fruits, called "swells" are roasted in coals until they burst and the skin is then peeled back and the sizzling pulp dipped in wood ashes and eaten. The fully ripe, fresh fruit is relished out-of-hand by children and adults, alike.

The pulp is made into a variety of products. It is an important ingredient in chutneys, curries and sauces, including some brands of Worcestershire and barbecue sauce, and in special Indian seafood pickle called "tamarind fish". Sugared tamarind pulp is often prepared as a confection. This sweetmeat is commonly found on the market in Jamaica, Cuba and the Dominican Republic. In Panama, the pulp may be sold in cornhusks, palm leaf fiber baskets, or in plastic bags.

Tamarind beverage, made from shelled fruits has long been a popular drink in the Tropics and it is now bottled in carbonated form in Guatemala, Mexico, Puerto Rico and elsewhere. Tamarind syrup is bottled for domestic use and export in Puerto Rico. In Mayaguez, street vendors sell cones of shaved ice saturated with Tamarind syrup.

The strained pulp, much like apple butter in appearance, can be stored under refrigeration for use in cold drinks or as a sauce for meats and poultry, plain cakes or puddings. Tamarind pulp can be made into a tart jelly, and Tamarind jam is canned commercially in Costa Rica. Tamarind sherbet and ice cream are popular and refreshing. In making fruit preserves, Tamarind is sometimes combined with guava, papaya or banana. Sometimes the fruit is made into wine. Young leaves and very young seedlings and flowers are cooked and eaten as greens and in curries in India. In Zimbabwe, the leaves are added to soup and the flowers are an ingredient in salads.

The oil extracted from the seeds is said to be palatable and have culinary quality. Tamarind seeds have been used in a limited way as emergency food. They are roasted, soaked to remove the seedcoat, then boiled or fried, or ground to a flour or starch. Roasted seeds are ground and used as a substitute for coffee. In Thailand they are sold for this purpose. (FAO, 2016)

2.9. Medicinal Uses:

In native practice, the pulp is applied on inflammations, used in a gargle for sore throat and, mixed with salt, as a liniment for rheumatism.

It is administered to alleviate sunstroke, digitalis poisoning, and alcoholic intoxication. The pulp is said to aid the restoration of sensation in cases of paralysis.

Tamarind leaves and flowers, dried or boiled, are used as poultices for swollen joints, sprains and boils. Lotions and extracts made from them are used in treating conjunctivitis, dysentery, jaundice, hemorrhoids and various other ailments, because of their antiseptics and vermifuges properties.

The fruit shells are burned and reduced to an alkaline ash, which enters into medicinal formulas. The bark of the tree is regarded as an effective astringent, tonic and febrifuge. Fried with salt and pulverized to an ash, it is given as a remedy for indigestion and colic. A decoction is used in cases of gingivitis and asthma and eye inflammations. Lotions and poultices made from the bark are applied on open sores and caterpillar rashes.

The powdered seeds and seeds coat are astringent and made into a paste for drawing boils and, with or without cumin seeds and palm sugar, are prescribed for chronic diarrhea and dysentery. An infusion of the roots is believed to have curative value in chest complaints and is an ingredient in prescriptions for leprosy.

Tamarind preparations are universally recognized as refrigerants in fevers and as laxatives and carminatives. Alone, or in combination with lime juice, honey, milk, dates, spices or camphor, the pulp is considered effective as a digestive, even for elephants, and as a remedy for bile disorders and for the treatment of scorbutic disease.

In Colombia, an ointment made of tamarind pulp, butter, and other ingredients is used to rid domestic animals of vermin. (Morton, 1987)

Chapter 3

Materials And Methods

MATERIALS AND METHODS

3.1. Plant material:

The plant was collected from Dhaka, Bangladesh.

3.2. Drying and grinding:

The collected plant parts (root and seed) were separated from undesirable materials or plants or plant parts. They were dried in the sun for one week after cutting into small pieces. The plant parts were ground into coarse powder with the help of a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

3.3. Preparation of the crude extract:

3.3.1. Cold extraction (Methanol extraction):

About 300 gm of powdered sample was taken in a clean, flat-bottomed glass container and soaked in 1500 ml of 90% methanol. The container with its contents was sealed and kept for a period of 10 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by apiece of clean, white cotton material. Then it was filtered through a filter paper.

3.3.2. Evaporation of solvent:

The filtrate was kept in an open space to evaporate the solvent thus crude extract was obtained.

- Fine powder of (*Tarmarindus indica*) Bark and Seed
- Dissolved in 90% Methanol
- Evaporation of solvent
- Crude extract

Fig 6: Rotary evaporator.

3.4. Phytochemical Screening Methods:

The plants kingdom represents reservoir of biological active compounds with various chemical structure and protective properties. The phytochemicals are often secondary metabolites present in smaller quantities in higher plants include include the alkaloids, glycosides, flavonoids, tannins, terpenoids and many other. Nearly 50% of the drugs used in medicine are of plants origin and only a small fraction of plants with medicinal activity has been assayed. The phytochemical isolated are then screened for different types of biological activity. (Evans, 2002) (Ghani, 2005)

3.4.1. Test Materials:

Extraction of Bark of ***Tarmarindus indic*a (L.)**

3.4.2. Reagents of chemical group tests:

- Mayer's Reagent
- Fehlings Solution II
- Dragendroff's Reagent
- Distilled water
- Fehling's Solution I
- Molish Reagent
- Ethanol
- Ferric chloride

3.4.3. Test for Glycosides:

1 ml solution of the extract was taken into a test tube. Few drop of Sodium .If a Yellow color form that shows the presence of glycosides.

3.4.4. Test for Alkaloids:

In testing for Alkaloids, about 1 ml of each extract will be stirred with 2.5 ml of I per cent aqueous hydrochloric acid on a water bath; 1ml of the filtrate is to be treated with a few drops of mayer's reagent and a second 1ml portion is to be treated the same way with Dragendorff's reagent. Orange-brown ppt. indicates the presence of alkaloid.

3.4.5. Test for Flavonoid:

A small quantity of test residue was dissolved in 1 ml of ethanol (95 % v/v) and treated with few drops of concentrated hydrochloric acid and 0.5 g of magnesium metal. If the pink, crimson or magenta color is developed within a minute or two that mean flavonoids are present.

3.4.6. Test for Tannins:

About 1ml of each portion of plant extract will be stirred with 10ml distilled water, filtered, and ferric chloride reagent will then be added to the filtrate. A blue-black, green or blue-green-precipitate is taken as evidence for the presence of tannins (Evans, 2002). Blue-black color is formed indicates the presence of tannin.

3.5 Antioxidant tests

3.5.1 Quantitative assay

Stock solution of the plant extract was prepared in ethanol from which a serial dilution was carried out to obtain concentration of 1, 5, 10, 50, 100, 500 µg/ml. Diluted solutions (2ml) were added to 3 ml of a 0.004% ethanol solution of DPPH, mixed and allowed to stand for 30 minutes for reaction to occur. The absorbance was determined at 517nm and from these values corresponding percentage of inhibitions were calculated. Then % inhibitions were plotted against log concentration and from the graph IC50 was calculated. The experiment was performed 3 times and average absorption was noted for each concentration.

3.5.2 Reagents

- Ethanol
- 0.004% DPPH (1 , 1 - diphenyl - 2 – picrylhydrazyl - hydrate)

3.5.3 Apparatus

- Test tubes and stands
- Beakers
- Pipettes
- Volumetric flasks
- UV spectrophotometer
- Electric balance
- Spatula
- Foil paper
- Sonicator
- Funnel

- Tissue paper
- Marker

3.5.4 Preparation 0.004% DPPH solution

4mg of DPPH was measured and dissolved in 100ml of ethanol thus 0.004% DPPH solution was prepared.

3.5.5 Procedure

- At first 6 volumetric flasks are taken to make 6 different types of concentration (1, 5, 10, 50, 100 and 500 µg/ml)
- Test tubes and volumetric flasks are rapped with foil paper.
- In 6 volumetric flaks serial dilution of extract is done and marked them respectively.
- 1ml of sample from each concentration and 3 ml of 0.004% DPPH solution is taken with the help of pipette in 6 test tubes respectively.
- Then solution is kept in dark place for 30 minutes with raping each test tube with foil paper.
- In another test tube 3ml 0.004% DPPH & 1ml methanol is taken to prepare blank solution.
- Then absorbance is taken by UV Spectroscopy.
- The percent of inhibition is calculated by using following formula -

$$\% \text{ inhibition} = \frac{\text{Blank absorbance} - \text{Solution absorbance}}{\text{Blank absorbance}} \times 100$$

3.6. Determination of Total Phenolics:

Total phenolic content of the different extractives of L. globosus were determined employing the method as described by Singleton in 1965 involving Folin-Ciocalteu reagent as oxidizing agent and catechin as standard.

3.6.1 Principle:

The content of total phenolic compounds of different fractions in the plant was determined by Folin–Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotungstates–molybdates. Sequences of reversible one or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})_4$. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI):

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

3.6.2 Materials:

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

3.6.3 Experimental procedure:

The amount of total phenolics in extract was determined according to the Folin-ciocalteu procedure. Samples (500µl) were introduced into test tubes. 2.5mL of Folincio-calteu reagent and 2.5 ml of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 30 min. Absobance at 760 nm was measured. The total phenolic content was expressed as Gallic acid equivalents (GAE) in milligrams per gram extract as calculated from standard Gallic acid graph by the following formula.

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

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

c = the concentration of gallic acid established from the calibration curve, mg/ml;
V = the volume of extract, ml;
m = the weight of different pure plant extracts, gm.

3.7. Determination of Total Flavonoids (TF):

Total flavonoid content of the different extractives of *L. globosus*was determined by aluminum chloride colorimetric method. Catechin was used as standard and the flavonoid content of the extractives was expressed as mg of catechin equivalent/gm of dried extract.

3.7.1. Principle:

The content of total flavonoids in different extractives of plant extract was determined by the well-known aluminum chloride colorimetric method. In this method aluminum chloride forms complex with hydroxyl groups of flavonoids present in the samples. This complex has the maximum absorbance at 510 nm.

3.7.2. Materials:

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

3.7.3. Experimental procedure:

Total flavonoid (TF) was determined using the procedure by Dewanto, Wu, Adom, and Liu, (2002). One milliliter of extract containing 0.1 g/mL of dry matter was placed in a 10 mL volumetric flask and then 5 mL of distilled water added followed by 0.3mL of 5% $NaNO_2$. After 5 min, 0.6 mL of 10% $AlCl_3$ was added. After another 5 min 2 mL of 1M NaOH was added and volume made up with distilled water. The solution was mixed and absorbance measured at 510 nm. TF amounts were expressed as catechin equivalents per dry matter. All samples were analyzed thrice and result averaged.

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

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

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.

3.8. In-Vitro Acetyl Cholinesterase Inhibitory Studies:

3.8.1. Principle:

The acetylcholinesterase inhibitory activity of different extractives, column subfractions and isolated compounds of L. globosus was determined by Ellman's method (Ellmanet al., 1961). This method estimates 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.

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

Thiocholine + dithiobisnitro $\longrightarrow$ Benzoate yellow color

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

3.8.2 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)
- BCA kit (bicinchoninic acid; Sigma Co., USA)
- Bovine serum albumin (Merck, India)
- Donepezil (Sigma-Aldrich, Japan)
- Micropipette (100-1000 µl)
- UV spectrophotometer (Shimadzu, USA)

3.8.3 Experimental Procedure:

The acetylcholinesterase (AChE) inhibitory assay was performed according to the colorimetric method of Ellmanusing acetylthiocholine iodide as a substrate. For the enzyme source, the rat brains were homogenized in a homogenizer with 5 volumes of a homogenation buffer [10 mMTris-HCl (pH 7.2), which contained 1 M NaCl, 50 mM $MgCl_2$ and 1% Triton X-100] and centrifuged at 10,000 rpmfor 30 min. The resulting supernatant was used as an enzyme source. All of the extraction steps were carried out at 4°C. Protein concentration was determined using the BCA kit (bicinchoninic acid; Sigma Co., USA) with bovine serum albumin (BSA) as a protein standard. The rates of hydrolysis by acetylcholinesterase were monitored spectrophotometrically. Each sample or standard (500 µl) was mixed with an enzyme solution (500 µl) and incubated at 37°C for 15 min. Absorbance at 405 nm was read immediately after adding an Ellman's reaction mixture [3.5 ml; 0.5 m Macetylthiocholine, 1 mM 5, 5´-dithio-bis (2-nitro benzoic acid)] in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. Reading was repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. The blank reaction was measured by substituting saline for the enzyme.

3.9. *In- Vitro* Butyryicholinesterase Inhibitory Studies:

3.9.1. Principle:

The butyrylcholinesterase inhibitory activity of different extractives, column subfractions and isolated compounds of L. globosus was determined by Ellman's method. This method estimates BChE using butyrylcholine iodide (substrate) and dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with dithiobisnitro benzoate ion. The color intensity can be measured on a spectrophotometer and the enzyme activity expressed as the rate of reaction per minute.

3.9.2. Materials:

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

3.9.3. Experimental procedure:

The butyrylcholinesterase (BuChE) inhibitory assay was performed according to the colorimetric method of Ellman using butyrylthiocholine iodide as a substrate. For the enzyme source, human blood are collected and centrifuged at 4000 rpm for 5 min. The resulting supernatant was used as an enzyme source. All of the extraction steps were carried out at 4°C. Then 50 µl enzyme, extraction buffer and plants extracts are incubated for 2 hours at room temperature. The rates of hydrolysis by butyrylcholinesterase were monitored spectrophotometrically. After 2 hours 200 µl DTNB (0.7mM) and 400 µl BTCI (0.35mM) added respectively. Heat this for 40 minutes at 37^0C. For measuring the background BTCI was avoided. Reading was taken at 412nm. From the difference between BTCI positive and negative data the activity of extract was measured. The blank reaction was measured by substituting saline for the enzyme.

Chapter 4

Results And Discussion

RESULTS AND DISCUSSION

4.1 Result of Phytochemical Screening:

4.1.1 Results of chemical group tests:

Results of the phytochemical screening of the Methanolic Extract of ***Tamarindus indica L.*** *(L.)*

Table 4.1: Phytochemical tests of *Tamarindus indica.*

Tested groups	**Methanolic Extract of *Tamarindus indica* L.**	
	Leaf	**Seed**
Steroids	–	–
Glycoside	–	–
Tannin	–	+
Alkaloids	+	+
Terpinoids	+	+

Note:

+ = Indicates the presence of the tested group,

− = Indicates the absence of the tested group.

The tests identify the presence of Alkaloids, Glycoside, Flavonoid, Saponin, Tannins, Reducing sugars, Gums in methanolic extract of *Tamarindus indica L.*

4.2. Result of Anti-oxidants test

4.2.1. Anti-oxidant Activity

Absorbance of Blank solution = 1.040

4.2.2. For Standard and Extract Solution

	Standard (Ascorbic acid)		Extract (leaf)		Extract (seed)	
Concentration	Absorbance (nm)	% of inhibition	Absorbance (nm)	% of inhibition	Absorbance (nm)	% of inhibition
1	0.989	4.9	0.842	18.97	0.952	8.39
5	0.887	14.71	0.872	16.12	0.910	11.57
10	0.710	31.73	0.739	28.88	0.901	13.35
50	0.148	85.77	0.454	56.34	0.270	80.08
100	0.101	90.29	0.139	86.59	0.084	91.85
500	0.081	92.21	0.097	90.65	0.105	89.88

Table 4.2: % of inhibition.

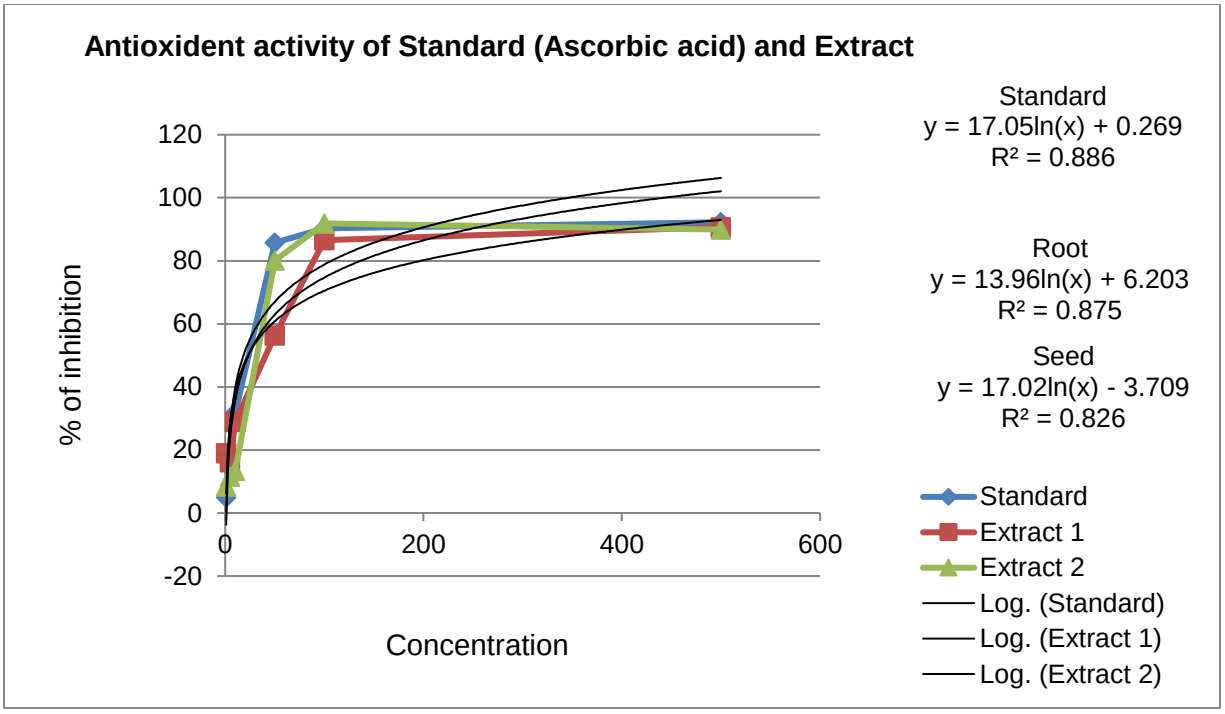

Fig 8: Graph of antioxidant activity.

Here we put concentration along X axis and % inhibition along Y axisy = 17.05ln(x) + 0.269, if y =50 then find out the value of x. That will be the IC_{50} value of standard ascorbic acid for DPPH was 18.35µg/ml and we put concentration along X axis and % inhibition along Y axis. y= 13.96In (x) + 6.203 and y= 17.02In (x) - 3.709, if y =50 then find out the value of x. That will be

the IC_{50} of the methanolic extract of *Tamarindus indica* L. Root was 23.10µg/ml and methanolic extract of *Tamarindus indica* L. Seed was 23.47µg/ml.

4.2.3. Results & discussion

The antioxidant activity of the methanolic extract *Tamarindus indica* L.was evaluated using DPPH free radical scavenging activity method. The methanolic extract of *Tamarindus indica* L. Seed and Seedhas excellent anti oxidant activity. The IC_{50} of the *Tamarindus indica*(L.) Root is 23.10 and µg/ml and *Tamarindus indica*(L.) Seed is 23.10 and µg/ml whereas IC_{50} of Ascorbic Acid is 18.35µg/ml.

4.3. Determination of Total Phenolics of Crude Methanol Extract (CME)

Phenolic content of the crude methanolic extract (CME) of was determined by 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 4.2 and in Figure 8. The results were expressed as mg of gallic acid equivalent (GAE)/gm of dried extract.

Table 4.3: 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

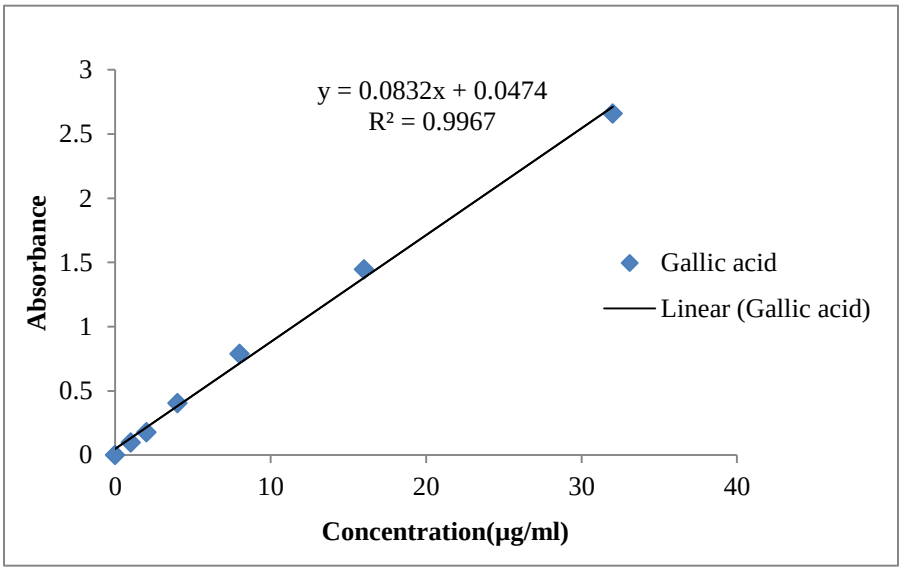

Fig. 9: Standard curve of gallic acid for the determination of total phenolics.

4.4. Determination of total flavonoids of crude methanol extract:

Total flavonoid content of the crude methanol extract (CME) and its four fractions (EAF, CLF, PEF, and AQF) 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 4.5 and in Fig.4.4. 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	0.231	0.226	0.235	0.231±0.003682
62.5	0.385	0.382	0.378	0.382±0.002867
125	0.736	0.742	0.749	0.742±0.005312
250	1.464	1.511	1.472	1.472±0.020532
500	2.732	2.741	2.738	2.737±0.003742

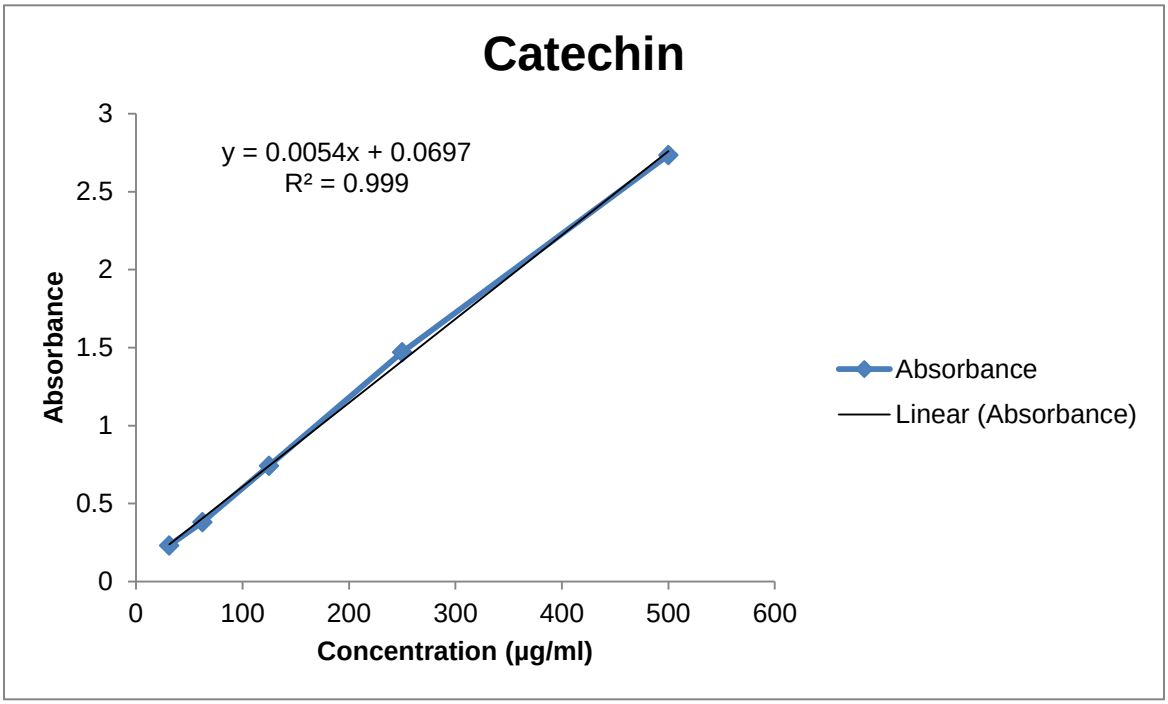

Fig. 10: Standard curve of catechin for the determination of total flavonoids.

The flavonoid content of the crude methanol extract (CME) and its different fractions were shown in Table 4.6 and Fig. 4.5.

The flavonoid content of CME was found to be 254.28 mg of CE/gm of dried extract, whereas the flavonoid content of EAF, CLF, PEF, and AQF was 444.33 mg, 81.51 mg, 106.75 mg, and 291.68 mg of CE/gm of dried extract, respectively. These results demonstrated that the ethyl acetate fraction (EAF) contained the highest amount of flavonoids among the CME fractions.

Table 4.5: Determination of total flavonoid content of the crude methanolic extract.

Sample	No. of sample	Conc. (↘g/ml)	Absorbance	CE/gm of dried sample	Mean ± STD
Crude metyhanolic extract (CME)	1	250	0.733	254.78	254.28 ± 1.78892
	2	250	0.729	252.29	
	3	250	0.736	255.76	

4.5. Acetylcholinesterase inhibitory activity assay:

Table 4.6: Determination of total acetylcholine content of the crude methanolic extract

Sample	No. of sample	Conc. (↘g/ml)	Absorbance	mg of GAE/gm of dried sample	Mean ± STD
Crude metyhanolic extract (CME)	1	100	1.296	75.24	75.55 ± 0.292803
	2	100	1.302	75.60	
	3	100	75.82	75.82	

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

Table 4.7: Acetylcholinesterase inhibitory activity of Donepezil, crude methanolic extract.

Name of sample	Conc. (µg/ml)	% of inhibition			% of inhibition Mean ⬅ STD	IC50 (µg/ml)
		a	b	c		
Donepezil (Std)	6.25	36.55	37.45	37.83	37.28 ± 0.53673	8.21
	12.5	63.35	63.87	63.65	63.62 ± 0.21312	
	25	78.34	78.93	79.11	78.79 ± 0.32887	
	50	86.45	86.86	87.30	86.53 ± 1.5805	
	100	89.36	90.64	91.85	90.62 ± 1.01667	
CME	12.5	6.31	7.02	6.77	6.70 ± 0.360	188.67
	25	14.23	15.76	14.86	14.95 ± 0.76896	
	50	28.46	28.00	28.43	28.30 ± 0.77861	
	100	44.23	44.38	44.56	44.39 ± 0.238868	
	200	52.15	52.46	53.11	52.57 ± 0.489932	

4.6.Butyrylcholinesterase inhibitory activity of enzymes:

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

The inhibitory activity of the crude methanol extract (CME) and its four different fractions against human plasma BuChE was assessed by the modified Ellman method using S-butyrylthiocholine as substrate and the results have been shown in table.

Table 4.8: Butyrylcholinesterase inhibitory activity of the crude methanolic extract

Name of the Sample	Conc. (µg/ml)	% of inhibition			Mean ± STD	IC50 (µg/ml)
		A	B	C		
CME	12.5	8.02	8.77	8.41	8.40 ± 0.3751	176.30
	25	16.53	16.67	16.91	16.04 ± 0.5802	
	50	30.18	30.11	30.77	30.67 ± 0.47056	
	100	43.19	43.49	43.67	43.78 ± 0.10275	
	200	52.72	52.82	52.11	52.55 ± 0.38431	
Galantamine (Standard)	**6.25**	38.55	38.78	39.11	38.81 ⬅ 0.28148	9.23
	12.5	66.35	65.67	66.67	66.23 ⬅ 0.51068	
	25	78.34	77.87	78.03	78.08 ⬅ 0.23895	
	50	85.45	85.02	84.33	81.93 ⬅ 0.14616	
	100	90.36	90.11	89.78	90.08 ⬅ 0.29091	

Chapter 5

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