MAPPING OF THE AMYLOIDOGENIC PEPTIDES INDUCED GENOTOXICITY IN RELEVANCE TO NEURONAL CELL DEATH

A Thesis submitted to the UNIVERSITY OF MYSORE in fulfilment of the requirements for the degree of

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in

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by

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CERTIFICATE

I, Ramesh BN, certify that the thesis entitled "**Mapping of amyloidogenic peptides induced genotoxicity in relevance to neuronal cell death**" is the result of research work done by me under the supervision of Dr. K.S.Jagannatha Rao at Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore. I am submitting this thesis for possible award of Doctor of Philosophy (Ph.D) degree in Biochemistry of the University of Mysore.

I further certify that this thesis has not been submitted by me for award of any other degree/ diploma of this or any other University.

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I hereby certify that the thesis entitled "**Mapping of amyloidogenic peptides induced genotoxicity in relevance to neuronal cell death**", submitted by Mr. Ramesh BN to the University of Mysore, Mysore, for the degree of Doctor of Philosophy is the result of research work carried out by him in the department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, under my guidance and supervision. This work has not been submitted either partially or fully for any other degree or fellowship.

Dr. K.S.Jagannatha Rao

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Dedicated to my Grandmother and beloved parents

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Mr. Ramesh, B.N.

ABSTRACT OF THE THESIS

Amyloidogenic peptides play an important role in neurodegeneration. Recently, there are reports on the nuclear localization of amyloids and proposed to have implications to DNA damage and neuronal apoptosis. The mechanism of amyloidogenic peptides induced neuronal cell death is still not clear. Our main questions are, (i) mapping of amyloidogenic peptides induced genotoxicity; (ii) Aβ(1-42) induced genomic instability in aged rabbits and (iii) to study anti-amyloidogenic properties of indigenous plants. To achieve these objectives we have employed agarose gel, Tm, EtBr binding, DNase I sensitivity assay, CD, MRI and TEM techniques. The results are summarized as follows: (i) Aβ fragments (1-11, 1-28, 1-40 and 1-42) nick scDNA and causes the open circular and linear forms. The Mg^{2+} ion enhanced the nicking activity of Aβ fragments (1-11, 1-28, 1-40 and 1-42). The modification of histidine of Aβ(1-11) abolished the DNA nicking activity. The DNA instability by studying Tm and EtBr binding revealed that DNA became unstable in presence of different fragments of Aβ. (ii) The aged (4 yr) New Zealand rabbits were intracisternally injected with Aβ(1-42) and DNA analyzed from aged rabbits for stability showed that DNA is damaged in FC and H; where as in M, DNA is in condensed state. The DNA conformation study evidenced the presence of C, π and ψ- type DNA conformations in FC, H and M of Aβ(1-42) injected (25 days) rabbit brain regions respectively. MRI studies showed no significant changes in brain structures between control and Aβ(1-42) injected aged rabbit brain regions. MRI scanning before and after 45 days of Aβ injection found significant reduction in the thickness of hippocampus, temporal lope, frontal lobe, and midbrain and an increase in lateral ventricle volume. Additionally, the aqueous leaf extracts of *C. crista* and *C .asiatica* could not prevent the DNA nicking activity of Aβ(1-42). The CG and VG induced modified conformations in Aβ(1-11), Aβ(1-28) and Aβ(25-35). The aqueous extracts of *C. crista* and *C. asiatica* showed anti-oxidant and anti-inflammatory activities. The *C. crista* showed anti-amyloidogenic properties.

Synopsis

Title of the thesis: Mapping of amyloidogenic peptides induced genotoxicity in relevance to neuronal cell death

Amyloidogenic peptides play an important role in neurodegeneration. These peptides are derived from the amyloid precursor protein through secretase activity. The neurotoxic effect of amyloidogenic peptides is manifested by alterations in metal homeostasis, oxidative stress, protein-protein interaction, apoptosis etc. The mechanism of amyloidogenic peptides induced neuronal cell death is still not clear. Recently, there are reports on the nuclear localization of amyloids and proposed to have implications related to DNA damage and neuronal apoptosis. The earlier studies clearly showed nuclear localization of amyloids and its binding to P53 promoter which may play a key role in novel pathogenesis related to apoptosis. Further, the exposure of human pre-neural NT2 cells to amyloids provoked up-regulation of DNA damage inducing gene GADD45. The *in vitro* studies showed that Aβ(1-42) nicks DNA and this observation provided new evidence on non-apoptotic mediated cell death. But mechanism of DNA damage caused by amyloids is still not clearly known. Our main questions are, (i) mapping of amyloidogenic peptides induced genotoxicity; (ii) Aβ(1-42) induced genomic instability in aged rabbits and (iii) to study anti-amyloidogenic properties of indigenous plants.

Objectives:

1. To study mapping of amyloids induced genotoxicity.
2. In vivo studies using aged rabbits to understand the neuroprotective role Indigenous plants in amyloids induced genotoxicity in relevance to brain tomography and DNA damage.
3. To study anti-amyloidogenic properties of indigenous medicinal herb

The thesis is divided in to five chapters.

Chapter 1: General introduction

This chapter begins with general information on Alzheimer's disease (AD) and its clinical and biological complexity. The description of etiological factors including genetics, environmental factors, and general lifestyles are discussed in detail. The hallmark pathologies such as senile plaques and intracellular deposits of the microtubule-associated tau protein as neurofibrillary tangles in AD have been described. The pathways of APP processing such as amyloidogenic and non-amyloidogenic pathways are described in detail. Brief account of enzymes involved in APP processing such as α- β- and γ - secretase and their role in amyloid production is highlighted. The neurotoxicity of Aβ through different mechanisms such as disrupting calcium homeostasis, inflammatory response, mitochondrial dysfunction, oxidative stress and genotoxicity are described. The role of diet in AD is discussed in detail. Also, the gene-diet interactions are highlighted. The protective dietary factors such as unsaturated fatty acids, vitamins, antioxidants, and polyphenols are explained elaborately. The introduction ends with future directions and objectives.

Chapter 2: To study mapping of amyloids induced genotoxicity.

Aβ is strongly implicated in AD. The genotoxicity of Aβ is not understood clearly. The available literature suggests that Aβ mediates DNA damage indirectly by oxidative mechanisms. But there are limited reports on the direct involvement of Aβ in genotoxicity. The reports showed that Aβ translocates to nuclear region and nicks DNA and behave as endonucleases. But the mechanisms are not clear. In the present study, we have provided data on Aβ fragments induced genotoxicity. To achieve this objective, we used different length Aβ fragments (1-11, 1-28, 22-35, 25-35, 17-42, 1-40, 1-42 and 1-43). Aβ induced DNA nicking is demonstrated using agarose gel electrophoresis. The results showed that Aβ fragments (1-11, 1-28, 1-40 and 1-42) nick scDNA and causes the open circular and linear forms. Aβ fragments (22-35, 25-35, 17-42 and 1-43) do not nick scDNA. In presence of Mg^{2+}, Aβ fragments (1-11, 1-28, 1-40 and 1-42) enhanced DNA nicking and while other Aβ fragments (22-35, 25-35, 17-42 and 1-43) also showed DNA nicking activity. To further understand the mechanisms, we hypothesized the involvement of histidine in DNA nicking is a factor. Aβ(1-42) has three histidine at 11^{th} , 13^{th} and 14^{th} positions. We selected Aβ(1-

11) since it has only one histidine at 11th position. The histidine in Aβ(1-11) is modified using diethyl pyrocarbonate method and assayed for its DNA nicking ability. The results showed that histidine modified Aβ(1-11) did not nick DNA. Additionally, histidine modified Aβ (1-11) retarded λ-DNA, single stranded circular and double stranded circular DNA mobility compared to unmodified Aβ(1-11). To further understand the nicking behavior of amyloids, we have used nuclease inhibitor, Aurin tricartboxylic acid (ATA). ATA inhibited only Aβ(1-42) induced DNA nicking. We further characterized Aβ induced DNA instability by studying melting temperature (Tm) and ethidium bromide (EtBr) binding. The results showed that DNA became unstable in presence of different fragments of Aβ as evidenced by changes in the Tm and EtBr. The conformation of different Aβ fragments is studied using Circular Dichroism and the results showed all the Aβ peptides are in random coil conformation. The Protscale analysis revealed contribution of different amino acids to secondary conformation of Aβ(1-43).

Chapter 3: *In vivo* studies using aged rabbits to understand the neuroprotective role Indigenous plants in amyloids induced genotoxicity in relevance to brain tomography and DNA damage.

Chapter 3A-a: Aβ(1-42) induced genomic instability in aged rabbit brain

DNA instability has been implicated in the pathogenesis of neurodegenerative disorders including Alzheimer's disease, Amyotrophic lateral sclerosis, Down's syndrome, and Parkinson's disease etc. The data on DNA damage indicates that the damage is associated with disease as a pathological event. However, the role of DNA damage as a major event in neurodegeneration is still not clear. Amyloid accumulation in the form of senile plaques is well established in AD brain. Amyloid is hypothesized to cause genotoxicity leading to neuronal cell death. There are studies to show that amyloid induces genomic instability and also alteration in DNA topology *in vitro*. Recent studies showed the nuclear localization of Aβ and its DNA binding ability. Most of these studies are related to in vitro models. But there are no *in vivo* studies to understand the Aβ(1-42) induced genomic instability. Further, the role of Aβ(1-42) in causing neurodegeneration through genomic instability is an unexplored area. In this chapter, we aim to answer three specific questions, a) Does Aβ(1-42)

injection in aged rabbits alter DNA stability? b) Does Aβ(1-42) alter the DNA conformation? and c) Does Aβ(1-42) alter the structural changes in the aged rabbit brain? These questions may likely to answer the possible role of Aβ in neurodegeneration through genomic instability. The aged (4 yr) New Zealand rabbits are intracisternally injected with Aβ(1-42) and are sacrificed after 25 days, when the rabbits developed AD like behavior. Genomic DNA is isolated from frontal cortex (FC), hippocampus (H) and midbrain (M) regions of Aβ(1-42) injected and control rabbit brain. The DNA stability parameters are analyzed. And the results showed that DNA is damaged in FC and H; where as in M, DNA is in condensed state. The DNA conformation study evidenced the presence of C, π and ψ- type DNA conformations in FC, H and M of Aβ(1-42) injected rabbit brain regions respectively. But in control rabbit brain, DNA is in B- conformation in all the brain regions studied. Magnetic resonance imaging (MRI) studies showed no significant changes in brain structures between control and Aβ(1-42) injected aged rabbit brain regions.

Chapter 3A-b: Inhibition of Aβ (1-42) induced DNA damage by Indigenous plant extracts

We further evaluated the protective role of curcumin and its derivatives against Aβ(1-42) induced DNA nicking. The results showed that curcumin derivatives did not protect Aβ(1-42) from DNA nicking. Additionally, the aqueous leaf extracts of *C. crista* and *C. asiatica* is tested for its anti-DNA nicking activity of Aβ(1-42). Results showed that both extracts could not prevent the DNA nicking activity of Aβ(1-42). The effects of CG and VG on the conformation of Aβ(1-11), Aβ(1-28) and Aβ(25-35) are studied. The results revealed that CG and VG induced modified conformations in Aβ(1-11), Aβ(1-28) and Aβ(25-35).

Chapter 3B: Aβ (1-42) induced brain MRI changes in aged rabbits resembles Alzheimer's disease

Alzheimer's disease (AD) is characterized by brain atrophy and the reduction in brain volume. Aβ deposition in the form of senile plaques is positively correlated with brain atrophy. There are no animal models to mimic the AD pathology. The present study is undertaken to understand whether Aβ induced brain atrophy in aged rabbit brain

resembles MRI changes in AD brain?. Ten aged (4 yrs) rabbits are injected with 100μg of Aβ(1-42) through intracisternal route and ten control aged rabbits are injected with saline. Rabbits are subjected to 1.5T MRI scanning before and after 45 days of Aβ injection. The time course of 45 days is chosen as rabbits developed AD like symptoms such as forward head tilting, hemiplegic gait, loss of appetite, isolation behavior, splaying of extremities and paralysis. We found that there are significant reduction in the thickness of hippocampus, temporal lope, frontal lobe, and midbrain and an increase in lateral ventricle volume. All these significant changes indicate that Aβ induced brain atrophy mimics AD brain atrophy.

Chapter 4: To study anti-amyloidogenic properties of indigenous medicinal herb

Chapter 4A: Anti-oxidant and anti-inflammatory properties of *C. crista* and *C .asiatica*

The oxidative stress and inflammation have been implicated in neurodegenerative disorders like Alzheimer's disease. The generation of reactive oxygen species, which is toxic, is a part of normal metabolism of biological system. The balance between production of reactive oxygen species and anti-oxidants is essential in biological system to prevent adverse effects of oxidative stress. There are number of plant extracts which showed antioxidant activities. In this chapter, role of antioxidant and anti-inflammatory activities of *C. crista* and *C. asiatica* is investigated. In this chapter, antioxidant properties of aqueous leaf extract of *C. crista* and *C. asiatica* was carried out using, DPPH assay, reducing potential assay and superoxide assay. The 5-lipoxgenase is one of the key enzymes involved in inflammation process. This enzyme provides a good i*n vitro* model to screen molecules that have anti-inflammatory ability. The anti-inflammatory activity of two extracts was demonstrated using 5-lipoxygense assays as 5-lipoxygense involved in inflammation. The results showed both the extracts dose dependently scavenged $DPPH^{\bullet}$ and the IC_{50} of *C. crista* less than *C. asiatica*. Both the extracts showed more IC_{50} compared to standard ascorbic acid. The results of superoxide assay showed that *C. asiatica* and *C. crista* dose dependently scavenged the superoxide anion radical. The IC_{50} of *C. asiatica* is lesser than *C. crista*. But both the extracts showed IC_{50} more than the standard, ascorbic acid. The results of reducing potential assay indicates that *C. crista*

has more reducing potential compared to *C. asiatica*. Both the extracts showed lesser reducing potential compared to standard, ascorbic acid. The results of 5-lipoxygense inhibition assay showed both the extracts dose dependently inhibited the enzyme and *C. crista* effectively inhibited compared to *C. asiatica.* The relevance of these observations is discussed.

Chapter 4B: Anti-amyloidogenic property of leaf aqueous extract of *C. crista*

Aβ can self assemble to form dimers, soluble oligomers, and protofibrils and diffuse plaques through multistep–nucleated polymerization. The self-assembling evaluation of Aβ *in vitro* will provide an opportunity to screen molecules for anti-amyloidogenic property. The prevention of the formation of oligomers and the fibrils from soluble monomers is of therapeutic significance for AD drug discovery. There are reports on the plants crude extracts or pure compounds having anti-amyloidogenic properties, but the mechanism of their therapeutic potential is still not clear. *C. crista* Linn (Syn C. bonducella [L.] Roxb.) belong to family Fabaceae, found abundantly in tropical and subtropical regions of Southeast Asia. The seed kernel of *C crista* contains Cassane- and norcassane-type diterpenoids. The stem and roots also known to contain new type of diterpenes. The seeds are traditionally used for anthelimintic, antimalarial, antipyretic, anti-inflammatory effects, but there are no scientific evidences recorded. In this chapter we aim to understand the effect of leaf aqueous extract of *C. crista* in the prevention of Aβ(1-42) aggregation. We have used three battery tests to understand the effect of *C. crista* in preventing the formation of, i) aggregates from monomers (ii) aggregates from oligomers and also (iii) dis-integration of preformed fibrils of Aβ. Results showed that *C. crista* aqueous extract could effectively found to inhibit the Aβ(1-42) aggregation both from monomer and oligomers. Also, the extract is able to dis-integrate the preformed fibrils. The anti-amyloidogenic property of *C. crista* may be attributed to the polyphenols present in the aqueous extract. We proposed a possible the mechanism of inhibition of amyloid fibril formation by water-soluble phenolic compounds.

Chapter 4C: Studies to understand the effect of *C. asiatica* on Aβ(1-42) aggregation in *vitro*

C. asiatica has been traditionally used in Asia to cure various ailments. The present study focused in evaluating *C. asiatica* or its anti- Aβ aggregation properties. The dried leaves of Indian penny wort (botanical name: *C. asiatica*) is mixed with milk and consume as memory improving and this is practiced traditionally in selected regions in India. There are studies on diverse effects of *C. asiatica* such as acetylcholinesterase inhibition, antioxidant, neuroprotection, and amyloid load reduction. However, there are no studies to understand whether *C. asiatica* prevents Aβ aggregation. In the current study, we have used Aβ(1-42), which is the most amyloidogenic peptide, for the formation of oligomers, protofibrils and fibrils. And also, we planned to map whether *C. asiatica* inhibits Aβ aggregation. We used aqueous extract of *C. asiatica* as it is traditionally used by local population in Western Ghats as brain tonic. The present chapter is focused on, i) whether the *C. asiatica* leaf aqueous extract prevent the formation of oligomers and aggregates from monomer? (Phase I: Aβ(1-42) + extract co –incubation), ii) Whether the *C. asiatica* aqueous extract prevent the formation of fibrils from oligomers (Phase II- extract added after oligomers formation) and iii) whether the aqueous extract dis-aggregates pre-formed fibrils (Phase III - aqueous extract added to matured fibrils and incubated for 8 days). The aggregation kinetics was studied using thioflavin-T assay and Transmission Electron Microscopy (TEM). Our results showed that *C. asiatica* aqueous extract could not significantly inhibit the Aβ aggregation either from monomer and oligomers and also not be able to dis-integrate the preformed fibrils.

Chapter 5:

Thesis ends with final summary and conclusion of the investigation.

The thesis has following significant findings

1. The role of risk factors in the onset of neurodegeneration in AD is discussed. The role of diet AD is elaborated (Chapter 1).

2. The present investigation is focused on Aβ fragments induced genotoxicity. To achieve this objective, we used different length Aβ fragments (1-11, 1-28, 22-35, 25-35, 17-42, 1-40, 1-42 and 1-43) and the results showed that Aβ fragments (1-11, 1-28, 1-40 and 1-42) nick scDNA and causes the open circular and linear forms. Aβ fragments (22-35, 25-35, 17-42 and 1-43) do not nick scDNA. In presence of Mg^{2+}, Aβ fragments (1-11, 1-28, 1-40 and 1-42) enhanced DNA nicking and while other Aβ fragments (22-35, 25-35, 17-42 and 1-43) also showed DNA nicking activity. The histidine in Aβ(1-11) is modified using diethyl pyrocarbonate method and assayed for its DNA nicking ability and the results showed that histidine modified Aβ(1-11) did not nick DNA. ATA inhibited only Aβ(1-42) induced DNA nicking. We further characterized Aβ induced DNA instability by studying melting temperature (Tm) and ethidium bromide (EtBr) binding. The results showed that DNA became unstable in presence of different fragments of Aβ as evidenced by changes in the Tm and EtBr (Chapter 2).

3. DNA stability and conformation are important in the life cycle of an organism. The DNA instability is postulated to be one of the risk factors for neuronal death in neurodegenerative disorders. Among all other risk factors, amyloid is one of the most important risk factor for neurodegeneration. Aβ(1-42) is implicated in Alzheimer's disease (AD). In the present aged (4 yr) New Zealand rabbits are intracisternally injected with Aβ(1-42) and are sacrificed after 25 days, when the rabbits developed AD like behavior. Genomic DNA is isolated from frontal cortex (FC), hippocampus (H) and midbrain (M) regions of Aβ(1-42) injected and control rabbit brain and analyzed. And the results showed that DNA is damaged in FC and H; where as in M, DNA is in condensed state. The DNA conformation study evidenced the presence of C, π and ψ- type DNA in conformations in FC, H and M of Aβ injected rabbit brain regions respectively. But in control rabbit brain, DNA is in B- conformation in all the brain regions studied. Magnetic resonance imaging (MRI) studies showed no significant changes in brain structure between control and Aβ(1-42) injected aged rabbit brain regions (Chapter 3A-a). The curcumin and its derivatives and *C. crista* and *C. asiatica* did not protect Aβ(1-42) induced DNA damage. The effects of CG andVG on the conformation of Aβ(1-11), Aβ(1-28) and Aβ(25-35) revealed that CG

and VG induced modified conformations in Aβ(1-11), Aβ(1-28) and Aβ(25-35) (Chapter 3A-b).

4.The aged (4 yr) New Zealand rabbits are intracisternally injected with Aβ(1-42). After 45 days of injection aged rabbit brains are subjected to MRI. The results showed that there are significant reductions in the thickness of hippocampus, temporal lope, frontal lobe, and midbrain and increased lateral ventricle volume. All these significant changes indicate that Aβ induces brain atrophy mimics AD brain atrophy (Chapter 3B).

5. Amyloid beta (Aβ) is the major etiological factor implicated in Alzheimer's disease (AD). Aβ(1-42) self assembles forming oligomers and fibrils via multiple aggregation process. The studies aimed to decrease Aβ levels or prevention of Aβ aggregation which are the major targets for therapeutic intervention. Natural products as alternatives for AD drug discovery are a current trend. The present study focused on anti-oxidant, anti-inflammatory and anti-amyloidogenic activities of aqueous extracts of *C. crista* and *C. asiatica.*

6. The aqueous extracts of *C. crista* and *C. asiatica* insighted the dose dependent antioxidant and anti-inflammatory activities (chapter 4A).

7.We focused to understand effect of *C. crista* leaf aqueous extract amyloid aggregation. The results showed that *C. crista* aqueous extract not only found to inhibit the Aβ(1-42) aggregation both from monomer and oligomers but also able to dis-aggregate the preformed fibrils (Chapter 4 B).

8. Further, our present study focused on, i) whether the *C. asiatica* leaf aqueous extract prevents the amyloid aggregation. The aggregation kinetics is studied using thioflavin-T assay and Transmission Electron Microscopy. The results showed that *C. asiatica* aqueous extract could not able to inhibit the Aβ aggregation both from monomer and oligomers and also could not able to dis-integrate the preformed fibrils (Chapter 4C).

The present investigation provides novel evidence on the role of Aβ in causing the genomic instability *in vitro* and *in vivo*. Aβ also induced significant changes in brain atrophy after 45 days of intracisternal injection of Aβ(1-42) resembling AD brain atrophy. The aqueous extracts of *C. crista* potentially inhibited the formation of amyloid fibrils from monomers and oligomers but also dis-integrated the pre-formed fibrils while aqueous extracts of *C. asiatica* did not do either. The potential role of Aβ in neuronal dysfunction has been evidenced.

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LIST OF ABBREVIATIONS

Aβ	amyloid beta
8 OHG	8-hydroxyl-2-deoxyguanosine
AD	Alzheimer's disease
Al	aluminium
APOE	apolipoprotein E
APP	amyloid precursor protein
ATA	aurin tricarboxylic acid
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
Ca	calcium
C.crista	Caesalpinia crista
C.asiatica	Centella asiatica
CD	circular dichroism spectroscopy
CG	curcumin glucoside
CNS	central nervous system
CSF	cerebrospinal fluid
Cu	copper
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I

DSBs	double stranded breaks
DTT	dithreitol
dsc	double stranded circular
EDTA	ethylenediamine tetra acetic acid
ER	endoplasmic reticulum
ERK	extra cellular signal-regulated kinase
EtBr	ethidium bromide
Fe	iron
H_2O_2	hydrogen peroxide
HD	huntington disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPTLC	high pressure thin layer chromatography
KCl	potassium chloride
MAD	moderate Alzheimer's disease
Mg	magnesium
$MgCl_2$	magnesium chloride
MRI	magnetic resonance imaging
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
NFTs	neurofibrillary tangles
PD	parkinson's disease

PS1	presenilin 1
PS2	presenilin 2
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
SAD	severe Alzheimer's disease
ScDNA	supercoiled DNA
SDS	sodium dodecyl sulphate
SPs	senile plaques
SSBs	single stranded breaks
ssc	single stranded circular
TAE	tris acetate EDTA
TCA	tricarboxylic acid
TEM	transmission electron microscopy
Thio-T	thioflavin-T
Tm	melting temperature
Zn	zinc

Chapter 1

General Introduction

Chapter 1 General Introduction

1.1. Introduction

Alzheimer's disease (AD) is the most common form of dementia and affects one in four individuals over the age of 85. AD has multiple etiological factors including genetics, environmental factors, and general lifestyles [Kalaria *et al*., 2008], and its hallmark pathology includes extracellular amyloid β protein (Aβ) deposition in the form of senile plaques and intracellular deposits of the microtubule-associated protein tau as neurofibrillary tangles in the AD brain [Lee, 2001]. The diagnosis of this disease is based on the characteristic idiopathic psychometric deficits upon clinical evaluation and further confirmed by post-mortem due to the presence of the characteristic lesions described above [Selkoe, 2001]. Aβ is produced by sequential proteolytic processing of a larger Aβ protein precursor (AβPP) by β-secretase to generate a large secreted fragment sAPPβ and a 99 aa cellular fragment – CTFβ – that includes Aβ, the transmembrane domain and the intracellular domain of AβPP [Wilquet and Strooper, 2004].

Recently, there has been increasing support for a role of diets in AD [Gasior *et al*., 2006; Lau *et al*., 2007; Dosunmu *et al*., 2007]. A number of dietary factors such as saturated fatty acids [Hooijmans and Kilian, 2008], higher calorie intake [Luchsinger *et al*., 2002], and excessive alcohol [Panza *et al*., 2008] have been reported to increase the risk of dementia and AD. In contrast, antioxidants, fish, methionine-rich proteins, and vitamins were identified as protective against the disease [Kalmijn *et al*., 1997]. Thus the global variation in diet may be linked to differential prevalence of AD [Grant, 1998]. Several cross-sectional studies suggest a relationship between particular nutrients and the presence of cognitive changes [Gasior *et al*., 2006; Lau *et al*., 2007; Dosunmu *et al*., 2007]. However, these need to be confirmed at an experimental level [Launer *et al*., 2004].

Interestingly, the same dietary pattern of risk and protection has been long accepted for metabolic syndrome and cardiovascular risk. In agreement Diabetes and hyperinsulinaemia are considered as important risk factors for AD [Strachan, 2003]. Among the other risk factors, depressive illness, traumatic head injury [Mayeux *et al*.,

1995], cardiovascular disease [Abellan *et al*., 2009; Luchsinger and Mayeux, 2004], smoking [Ott *et al*., 1998; Merchant *et al*., 1999], and stroke [Honing *et al*., 2003; Vermeer *et al*., 2003] are significant issues that may be related to the same risk factors. Further, the ε4 allele variant of apolipoprotein E (ApoE) has been associated with increased risk in sporadic and familial AD [Mayeux *et al*., 1993]. As a carrier of cholesterol, ApoE-ε4 is also a risk factor in cardiovascular disease.

Oxidative stress has been suggested to play a major role in the pathology of AD [Aksenov *et al*., 2001; Markesberry, 2003; Markesberry and Carney 1999; Sayre *et al*., 2001], and evidence for its mediation includes: 1) generation of free radicals through metal ions; 2) enhanced lipid peroxidation; 3) increased DNA and protein damage; and 4) increased tau protein phosphorylation [Sayre *et al*., 2001]. The human body has a defense mechanism to cope with oxidative stress, or prevent the onset of oxidative stress, through endogenous antioxidants derived from enzymatic or non-enzymatic sources [Buterfield *et al*., 2002], namely superoxide dismutase, glutathione reductase, and catalase. The nonenzymatic sources of endogenous antioxidants include glutathione, uric acid, α-lipoic acids, acetyl L-carnitine, melatonin, and dehydroepiandrosterone. Endogenous antioxidants also include the products of reactions catalyzed by enzymes that are up regulated in response to oxidative stress (e.g., bilurubin) [Buterfield *et al*., 2002]. Dietary antioxidants and metal chelators were found to be associated with a reduced AD risk further reinforcing the importance of dietary stress on the body's homeostasis pathways and nutritional guidelines for AD prevention.

Several studies demonstrate that diet has a definite long-term effect on general health [Vermeer *et al*., 2003; Leibson *et al*., 1997]. Although several cross-sectional studies have indicated that diet plays a role in AD, the long time taken for the pathogenesis of AD makes it nearly impossible to experimentally demonstrate the specific effects of diets in AD. In this review, we attempt to define the action of presumably protective and harmful dietary habits on pathways identified as relevant to AD pathogenesis. A common theme that has emerged from the analysis is that aging and AD are associated with excesses in saturated lipids, homocysteine, oxidative stress and other toxic pathways that appear to result from reduced efficiency of clearance pathways in general. Thus, a general rule of AD is that moderation is key to disease prevention.

However, literature also suggests that several specific additions to the di*et* so help in AD prevention.

1.2. Role of Aβin AD

1.2.1. Aβ secretion pathways

Aβhypothesis is strongly proposed as a risk and pathological mechanism for neurodegenation in AD. Many drugs are targeted to reduce amyloid load in AD. But there is no unified hypothesis for AD neurodegeneration. Amyloid hypothesis is still under debate. Aβ peptides are produced by proteolysis of βAPP by secretases in neuronal cells. βAPP is trans-membrane protein which translocates to cell membrane from cytoplasm after translation. The processing of βAPP follows two pathways mediated by three secretary enzymes. One pathway is referred to as amyloidogenic, mediated by β-secretase and γ-secretase resulting in the excess production of Aβ40 Aβ42. Aβ is produced by sequential proteolytic processing of a larger Aβ protein precursor (AβPP). The β-secretase cleave at N-terminal Aβwhile γ -secretase cleave at c-terminal of Aβ.The cleavage of APP secretases results variants of Aβ namely Aβ(40)/Aβ(1-42). The activities of these two secretases on APP to generate a large secreted fragment sAPPβ and a 99 aa cellular fragment – CTFβ – that includes Aβ, the transmembrane domain and the intracellular domain of AβPP. The other pathway is non-amyloidogenic, mediated by α-secretase and γ -secretase. The cleavage site of α-secretase in APP is within Aβ and cleavage within Aβ results in prevention from forming full length Aβ. The N-terminal fragment generated by cleavage by α - or βsecretase is called as secreted APP (sAPP)α or APP (sAPP)β respectively. The carboxy-terminal fragments (CTF) generated by α- and β secretase are CTF83 and CTF99, respectively. The γ-Secretase cleavage of CTF83 and CTF99 generates p3 and Aβ, respectively and also forms amino-terminal APP intracellular domain (AICD) [Venugopal *et al*., 2008]. The sAPPαwill be produced when α- secretase act on APP. The function of sAPPαis not clearly understood so far. But in *vitro* studies suggest that it protects the neurons against oxygen-glucose deprivation and excitotoxicity and promotes neurite outgrowth, synaptogenesis and cell adhesion [Mattson *et al*., 1993; Furukawa *et al*., 1996; Mattson, 1997; Gakhar-Koppole *et al*., 2008]. But still no clear

biologic role has been established for the P3 fragment that is generated by α- and γ-secretase cleavage of APP. The sAPPβis the cleavage of APP by β-secretase. The biological functions of sAPPβis poorly understood. But recent report suggests that sAPPβ is involved in development of central and peripheral neurons [Nikolaev *et al*., 2009]. The APPβ also reported to suppress the neuronal stem cell differentiation [Kwak *et al*., 2006]. The carboxy-terminal fragments CTF83 and CTF99 are generated as result of cleavage by α-and βsecretase respectively. But these c-terminal fragments have no biologically relevant functions [Kwak *et al*.,2006]. The α-secretase activity is associated with one or more enzymes that belongs to metalloproteinase domain proteins (ADAM) [Asai *et al*., 2003; Tanabe *et al*., 2006]. Among this group, ADAM 9, 10, 17 and 19 are known to posses α-secretase activity. Currently, it is not clear on the involvement of ADAM in AD. But in mouse model, over expression of ADAM10 is associated with reduction in Aβ production, deposition and in turn lessening cognitive deficits [Postina *et al*., 2004]. It is hypothesized that ADAM10 may be a candidate for α-secretase in AD pathology. It may be also possible that more than one ADAM may be involved in generating sAPPαAPP is one among many substrates for ADAM family proteins. The other substrates include N-cadherin [Kohutek *et al*., 2009], EGFR ligands, TGF-α, notch and ephrin (Edwards *et al*., 2008; Le Gall *et al*., 2009). These molecules are known to enhance α-secretase. EGCG is one of the molecules, which is known to enhance activity of α-secretase and to increase frequency of non-amyloidogenic pathway [Levites *et al*.,2001]. The βsecretase activity is mediated by beta site APP cleaving enzyme 1(BACE1) which is major β secretase in the brain [Vassar *et al*., 1999]. A related enzyme BACE2 is mostly expressed in glial cells and also known to cleave APP. BACE2 expression levels are low in brain [Laird *et al*., 2005]. The genetic ablation of BACE1 is known to prevent the amyloid pathology in mouse model expressing Swedish mutation of APP (APP670/671) with mutated PS1. There are increased levels of expression of BACE1 in AD patients (Holsinger *et al*., 2002; Yang *et al*., 2003). The other substrates of BACE1 apart from APP are APP –like proteins 1 and 2 (APLP1and 2) voltage gated sodium channel β2-submit, low density lipoprotein related protein, neuregulin 1, P-selectin glyco-protein ligand-1, and sialyltransferase

ST6Gal I [Hunt and Turner 2009]. Recently reports showed that cathepsins have β secretase activity [Schechter and Ziv 2008; Bohme *et al*., 2008; Klein *et al*., 2009]. Additionally, cathepsin- B known to degrade Aβ[Muller-Steiner *et al*., 2006]. But these results are not conclusive and needs further investigation. BACE1 is an aspartyly protease that embedded in membrane which is essential in releasing the Aβfrom APP. The generation of Aβ results in the over accumulation of Aβ in the form of oligomers, protofibrils and fibrils which is the hallmark pathology of the AD [Cizas *et al*., 2010)]. But detailed understanding of function and regulation of BACE1 is essential before inhibiting its activity. The BACE1 knockout mice have shown myelination difficulties and the over-expression of BACE1 results in neurodegeneration. Venugopal *et al*. [2008] suggested that thorough understanding of the evolution of the BACE1 substrates is necessary in directing the drugs that inhibit the BACE1 which have implications to AD. The γ- secretase known to cleave APP in its intra-membrane region at γ- cleavage site to generate Aβ(40)/ (1-42) or p3 and AICD59/57, a second cleavage at the ε- cleavage site results in AICD50. γ- secretase is a complex protein of four proteins PS1 or PS2, Nct, Aph-1 and Pen2 [Wolfe, 2008]. PS1 and PS2 have the actual protease activity. PS1 is dominant presenilin in the brain. The formation of active complex of γ- secretase is as follows: First Nct and Aph-1 form complex, then PS binds to this complex. Finally Pen2 will complete the complex and may facilitate the auto cleavage of PS [Li *et al*., 2009]. The substrates for γ-secretase other than APP include, Notch, N-cadherin, p75NTR and neuregulin binding partner ErbB4 [Beel and Sanders, 2008; Wakabayashi and de Strooper, 2008]. Interestingly, γ- secretase has been implicated in activating non-neuronal cells such as astrocytes and microglia during brain injury [Nadler *et al*., 2008]. This study shows the involvement of γ- secretase in repair mechanisms in brain. These findings will shed light on the novel role of γ- secretase. Further studies are necessary to validate these findings. Thus there are complex mechanisms involved in the neuronal cell death in AD as indicated below

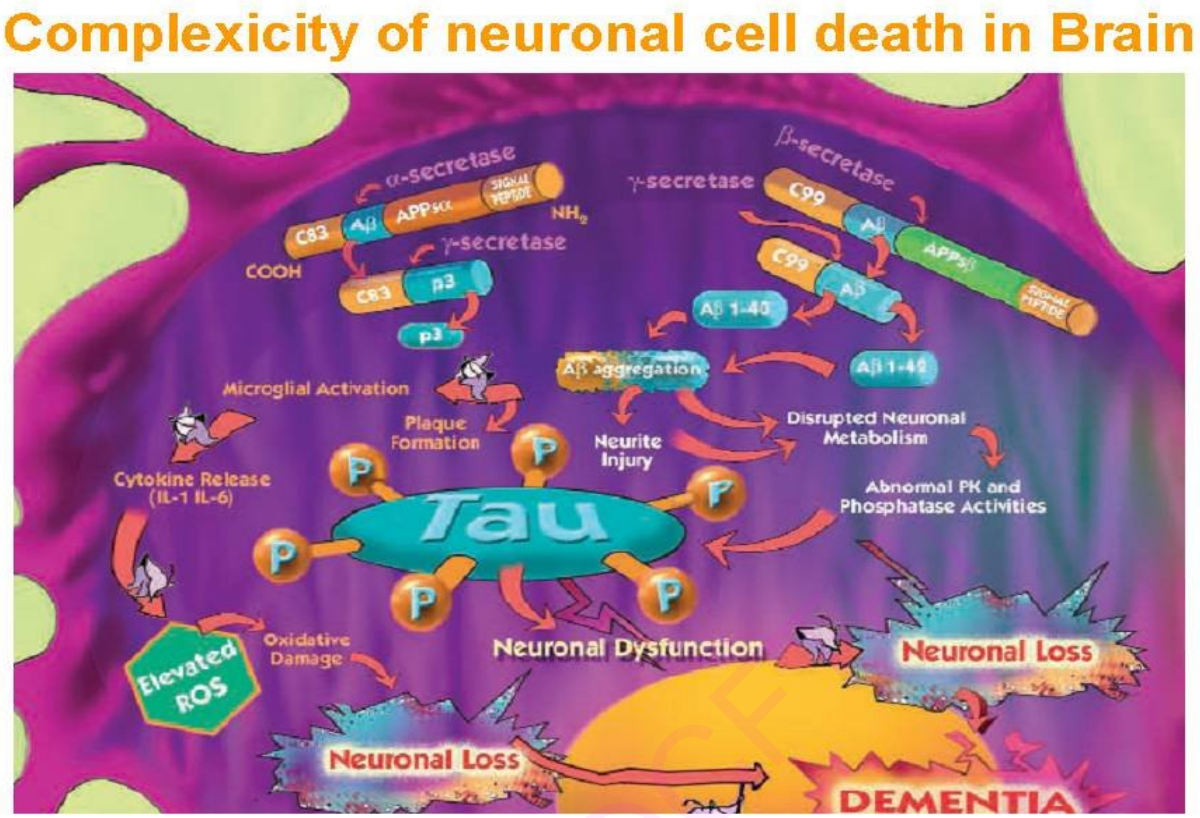


Fig 1.1: Complexities of different pathways of neuronal dysfunction and neuronal loss in Dementia and Alzheimer's disease: The beta-amyloid will be released from APP by the secretases activity leading accumulation of beta-amyloid into plaques. These plaques causes : (i) elevated the reactive oxygen species leading to oxidative damage and neuronal loss; (ii) neurite injury and phosphorylation of tau which causes neuronal dysfunction and (iii) activation of microglia and inflammation leading to neuronal loss. These pathways lead to dementia and Alzheimer's disease [Coutesy of National Institue on Aging website]

1.2.2. Aβload in AD

The accumulation of Aβ in the form of senile plaques is found to correlate with neuronal loss and brain atrophy and ventricular expansion [Hardy and Higgins, 1992; Silbert *et al*., 2003]. The brain atrophy is correlated with cognitive decline in AD. The studies with AD patients showed a positive correlation between amyloid load and brain atrophy [Archer *et al*., 2006; Silbert *et al*., 2003]. The significant contribution towards *in vivo* quantification of amyloid came through the development of Benzathiol derivative, N-methyl [11C] 2-(4'-methyl aminophenyl)-6-hydroxy-benzothiazole (PIB), which specifically bind to amyloid and can be seen in Positron

Emission Tomography (PET) scan [Klunk *et al*., 2001; Mathis *et al*., 2002; Wang *et al*., 2002]. The animal studies confirm that PIB crosses blood brain barrier [Bacskai *et al*., 2003]. The specificity of PIB is that it only binds to aggregated forms of Aβ and not to monomeric forms of Aβ [Klunk *et al*., 2003]. The results of two-year follow up study of amyloid deposition in AD patients suggest that amyloid load is high and stable throughout. But there is a decline in regional cerebral metabolic rate for glucose and cognitive function [Engler *et al*., 2006]. This indicates that amyloid deposition in AD patients is a dynamic process and reaches equilibrium very early in the course of AD. These kinds of study will be useful in evaluating drugs for AD. There should be some correlation between amyloid load in brain and in CSF. The decrease in the levels of Aβ(1-42) in CSF is reported in AD [Hampel *et al*., 2004; Riemenschneider *et al*., 2002]. The decrease in levels of Aβ(1-42) in very early phase of AD makes it a good biomarker for early detection of AD. The mechanisms by which Aβ levels decrease is not clear. However it is hypothesized that Aβ are sequestered in brain leading to reduced Aβ levels in CSF [Weller, 2001]. The above hypothesis is supported by increase in senile plaques which is in correlation with reduced Aβ levels in CSF [Strozyk *et al*., 2003]. Unfortunately, lower Aβ levels in CSF is found in other neurodegenerative disorders such as Creutzfeldt-Jakob disease, amyotrophic lateral sclerosis and multiple sclerosis [Blenow and Hampel, 2003]. This study revealed that Aβ levels in CSF is not specific to discriminate between AD and other neurodegenerative disorders. The possible explanation is provided by Sottibundu *et al*. [2008], where it is shown that soluble Aβ could able to reduce γ-secretase activity which leads to lesser production of Aβ. The qualitative measurement of Aβ may not be sufficient to discriminate patients having AD or not. So, measurement of amyloid load is necessary. Grimer *et al*. [2009] reported an inverse linear correlation between PIB uptake and CFS Aβ level in AD patients. Additionally, Grimer *et al*. [2009] reported that PIB uptake and CSF levels of Aβ found to correlate strongly in white and grey matter atrophy. This indicates that CSF levels can be considered as peripheral biomarker of amyloid pathology. There are reports linking the amyloid load and APOE in AD. The APOE is a genetic risk factor for sporadic AD [Farrer *et al*., 1997]. But the mechanism by which APOE is involved in AD is not clear. However, APOE is reported to have effect on APP metabolism via cholesterol

mediation, increase in amyloid aggregation and the neurotoxicity and decrease in the amyloid clearance [Koudinov *et al*., 1998; Holtzman, 2001; Yang *et al*., 1999]. The transgenic murine brain showed correlation between the increased expressions of APOE to amyloid load where as human APOE expression showed inverse correlation to amyloid load [Bales *et al*., 1997; Holtzman *et al*., 1999]. Lambert *et al*. [2005] reported that increased expression levels of APOE mRNA increased the amyloid load in AD patients. This is attributed to the polymorphism of APOE promoter. Interestingly, risk of APOE to AD decreases after age of 65 to 70 years [Farrer *et al*., 1997; Champagne *et al*., 2003]. Recently, Casellia *et al*. [2010] has reported that non-demented APOE carriers above 60 years have higher amyloid load in than non-carriers of APOE. However, no difference is observed regarding the NFT pathology between APOE carriers and non- carriers. The reduction of amyloid load is known to improve the cognition and memory at least in transgenic mice AD models. There are approaches to reduce the amyloid load that includes, reduction of amyloid production, preventing the amyloid aggregation and enhancing the amyloid clearance. These approaches have been discussed in the other sections. The unconventional approaches to reduce the amyloid load have been reported. The unconventional methods include life style and diet. The physical exercise is known to have positive effects on human body and is well-established fact. Interestingly, TgCRND8 mice subjected to exercise for five months reduced the amyloid load in frontal cortex and also in hippocampus [Adlard *et al*., 2005]. The reduction in the amyloid also correlated to enhanced learning of TgCRND8 mice. This data indicates that life styles have a strong influence on the onset of AD like pathology. The amyloid plaques near neurons are presented below.

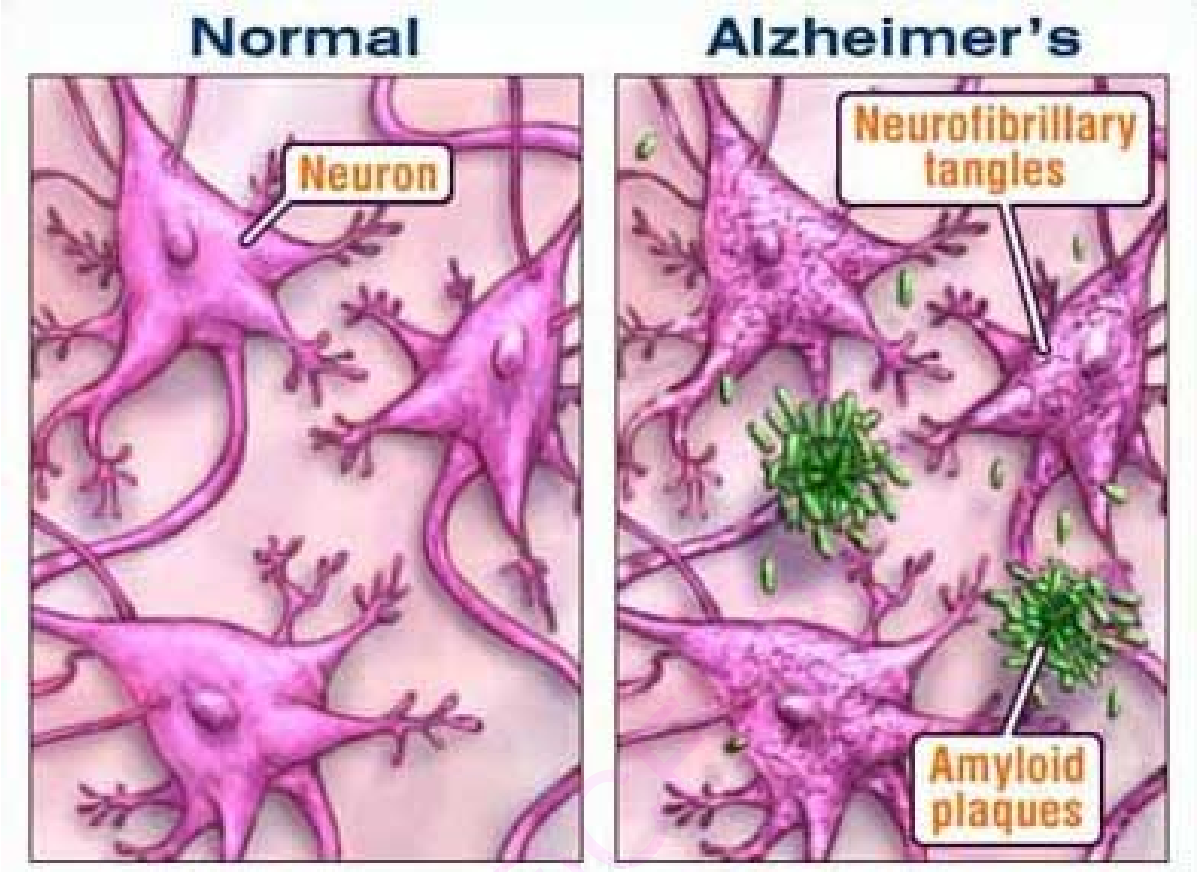


Fig 1.2: Neurons showing intracellular neurofibrillary tangles and extra cellular amyloid plaques (Courtesy from www. Healthyfellow.com/188/ alzheimers-disease).

1.2.3. Aβ induced neurotoxicity

The toxicity of Aβ depends on form of Aβ ie whether oligomer or fibril form. The recent understanding on amyloid hypothesis suggests that oligomeric form of Aβ is more toxic than fibrils. The neurotoxic effects of Aβ can be divided into three sections, Aβ effects on i) Free radical accumulation, ii) Altered calcium homeostasis and iii) inflammatory response. i) Free radical accumulation: The Aβ generates free radical by the mechanism, which is not clear. The possible mechanism is that it Aβ gets inserted into neuronal and glial membrane bilayer and generates oxygen dependent free radicals. The free radicals once generated attack lipid and proteins, causes lipid peroxidation and protein oxidation respectively [Varadarajan *et al*., 2000]. Aβ is known to induce production of H_2O_2 in cultured hippocampal neurons [Mattson *et al*., 2000] and in neuroblastoma cultures [Behl *et al*., 1994]. The oxidative

modifications of proteins in AD include advanced glycation end products (AGEs) and AGEs are known to increase DNA oxidation and membrane lipid peroxidation [Halverson *et al*., 1990]). ii) Altered Calcium homeostasis: There are many reports which claim that the Aβ disrupt cellular ion homeostasis. Aβ toxicity is mediated via altering the calcium influx by forming pore formation and also potentiation of calcium channels [Mattson *et al*., 1992; Vitek *et al*., 1994; Weiss *et al*., 1994]. The neurons exposed to Aβ showed sensitive to excitatory aminoacids and membrane depolarization [Mattson *et al*., 1992; Weiss *et al*., 1994; Mattson, 1994]. Calcium is an important messenger in brain and it also involved in neuronal development, synaptic transmission, neuronal plasticity and regulation of many metabolic pathways. There are reports on the association of senile plaques and neurofibrillary tangles with altered cellular calcium homeostasis both in cell culture models and in AD patients [Mattson and Chan, 2001]. iii) Inflammatory Response: The activated microglial cells and reactive astrocytes are found to be associated with neuritic plaques. These represent the inflammatory response mediators in AD [Chong *et al*., 2001]. Immune activation and inflammatory markers are significantly elevated in AD brains compared to age matched controls [Dumery *et al*., 2001]. It is reported that microglia from human AD brain exposed to Aβ found to secrete inflammatory mediators such as cytokines, complements and chemokines [Lue *et al*., 2001]. *In vitro* studies suggest that Aβ induce inflammatory response in AD [Lue *et al*., 2001]. The literature on genotoxicity of amyloids is scant. The available reports on genotoxicity of amyloids suggest that amyloids translocate to nuclear region [Gouras *et al*., 2000; Grant *et al*., 2000]. Hegde *et al*. [2004] has reported the presence of Aβ in nuclear region of AD brain sample and interaction with DNA has resulted in change in DNA conformation (Hegde *et al*., 2004). Anitha *et al*. [2002] also reported that DNA from hippocampus of AD patient in Z-DNA conformation. Buckig *et al*., [2002] has reported presence of aggregated Aβ(1-42) in the neucleus of CHO cells. The role of Amyloids in nucleus was not known. So, *in vitro* studies suggests that Aβ(1-42) cause DNA damage by nicking [Suram *et al*., 2007]. *In vitro* studies suggest that low concentrations of Aβ cause neuronal apoptosis with DNA condensation [Zeng *et al*., 2004; Blanc *et al*., 1997]. Yu *et al*. [2007] reported that Aβ(40) time dependently condensed the DNA *in vitro* and suggests that it is one of the mechanism of Aβ(40) induced genotoxicity. A

surface plasmon resonance study showed that all the soluble forms aggregates of Aβ(1-42) bind linear double stranded calf thymus DNA [Barrantes *et al*., 2007]. The role of Aβ(1-42)-DNA interaction is less understood. The general mechanisms of toxicity suggested include: oxidative stress [da Silva *et al*., 2005], production of toxic species [Ribe *et al*., 2005; da Silva *et al*., 2005] and apoptosis induction [Ohyagi *et al*., 2005]. The intra-cellular Aβ(1-42) binds and activates p53 promoter, which results in neuronal cell death [Ohyagi *et al*., 2005]. The above information on nuclear localization of Aβand its binding to DNA property and alteration of DNA conformation will gives an alternate pathways to AD neurodegeneration. The following MRI images shows the brain atrophy.

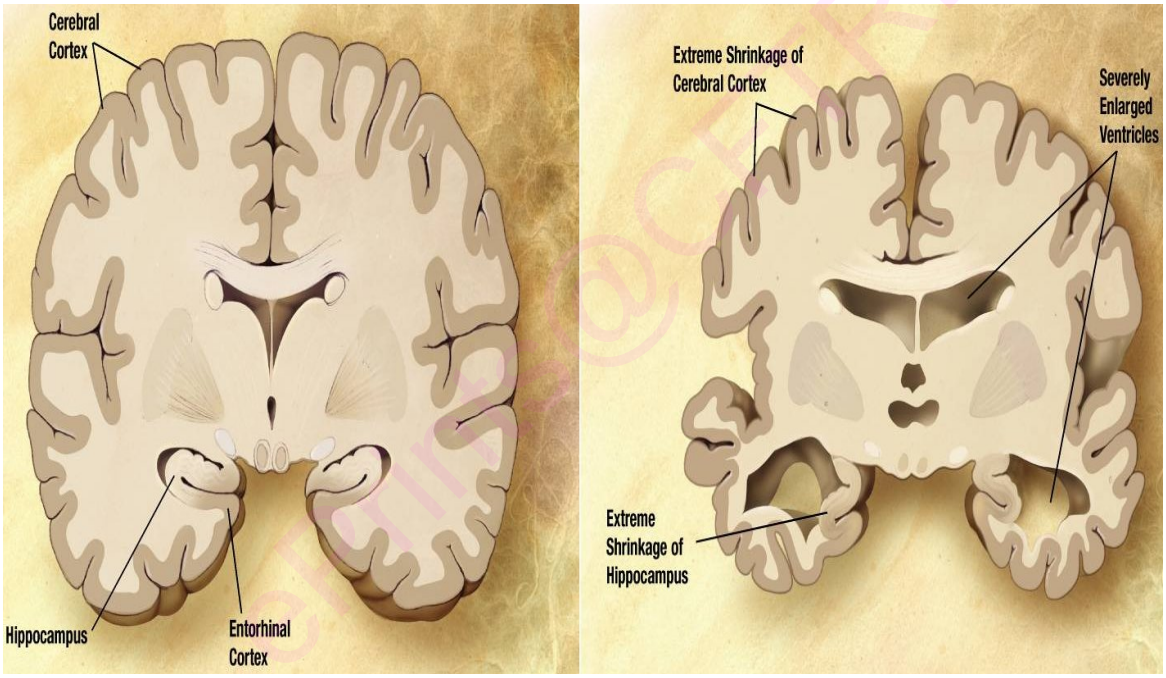


Fig 1.3: MRI images of normal and AD brain showing thickness and volume differences: The AD brain shows increased atrophy and increased lateral ventricle volume compared to control (Coutesy of Federation of American Socities For Experimental Biology).

1.2.4.Aβ as drug target

The currently available FDA for AD approved drug includes tacrine, donepezil, rivastigmine and galantamine and their use is restricted to USA. These drugs are

acetyl-cholinesterase inhibitors, which act indirectly on AD pathology. Aβ has been the main target of drug discovery in AD. There is a three lines of targeting the Aβ; i) reducing its production by altering or inhibiting the APP processing pathways [already covered in previous section], (ii) Abolishing or inhibiting aggregation and (iii) enhancing the clearance of Aβ which is previously accumulated. These strategies are presently employed to slow the progression of AD. The molecules that interact with Aβ and reduce its aggregation and accumulation there by preventing amyloid induced synaptic dysfunction and neurotoxicity is of therapeutic significance [Rafii and Aisen, 2009]. Tramiprosate is the first ant-amyloidogenic drug that reached pivotal clinical trails [Gervais, 2004]. It is a glycosaminoglycan mimetic that binds Aβ monomers, not only prevents the formation of aggregates but also and enhance the clearance of preformed Aβ aggregates there by prevents neurotoxicity [Gervais, 2004]. In phase II, Tramiprosate decreased Aβ(1-42) levels in CSF of AD patients [Aisen *et al*., 2006]. Phase II trials for Tramiprosate is conduced in North America by including 1052 patients with mild to moderate AD. These patients are randomly given placebo or 150 mg or 100mg of Tramiprosate twice in a day. Even though the patients well tolerated for the drug, but it fails to demonstrate beneficial effects such as improvement in cognitive function. The reasons for the failure of the study are attributed to unexplained variance. So, further trails are not planned based on this mechanism. Scyllo-inositol is another anti-amyloid aggregation drug that has shown promising results in clinical trials [McLaurin *et al*., 2000]. Another important lines of targeting Aβ(1-42) is immunotherapy. The antibodies that bind amyloid in blood and draw them form the brain through blood brain barrier by receptor mediation. The animal studies have shown that heparin and gelsolin are thought to trap Aβ and reduce Aβaccumulation in the brain [DeMattos *et al*., 2002]. In phase II trail of first generation amyloid vaccine AN-1792 (Elan/ Wyeth), active immunization is done using an aggregated Aβas immunogen. The results showed that AD patients developed positive immune response and continued over one year. Additionally, those AD also showed improvement in psychological tests [Gilman *et al*., 2005]. But the study is discontinued because 6% of the AD patients developed aseptic meningoencephalitis. The second-generation vaccine ACC-001 (Elan/Wyeth), having acceptable safety profile is used. This immunogen is a short Aβ sequence which is

likely to prevent induction of toxic cellular immune response. Phase II trial is ongoing and results are awaited. The passive immunization known to offers more efficacy and safety compared to active immunization. Because, passive immunization incorporates monoclonal anti- Aβ antibodies intravenously. One such monoclonal antibody, ineuzumab (Elan/ Wyeth) has undergone phase II trails and the results are encouraging and phase III trails are just launched.Aβ is known to cause inflammation and neuronal cell death via binding to receptors for advanced glycated end products (RAGE) (Chen *et al*., 2007). Experimental results showed that blocking Aβ- RAGE binding by RAGE–antagonist, PF-04494700 found to decrease amyloid accumulation and neurotoxicity [Chen *et al*., 2007]. So, RAGE–antagonist, PF-04494700 is presently undergoing phase II clinical trails to determine its potential for AD therapy. The decreasing the Aβ production by inhibiting γ- secretase: among the approaches to reduce Aβ levels in brain reducing its production is major approach. γ- secretase cleaves APP at one end of Aβ sequence and its activity is indispensable for Aβ generation in brain. So, inhibiting γ- secretase will reduce Aβ generation. Terenflurbil is inhibitor of γ- secretase activity and it is a enanatiomer of flurbiprofen [an non steroidal anti-inflammatory drug]. Terenflurbil is well tolerated in phase II clinical trials involving mild to moderate AD. But they did not show beneficial effect on cognitive function [Wilcock *et al*., 2008]. Other γ- secretase inhibitor that is under phase II study is semagacestat (LY450139). This has shown inhibition of γ-secretase activity, which resulted in significant reduction in amyloid production in blood and CSF [Fleisher *et al*., 2008]. But inhibtion γ- secretase activity also inhibit notch cleavage which is a cause of concern [Wolfe, 2008]. The two major pathological features in AD include senile plaques and neurofibrillary tangles. The therapeutics for AD should include targeting both amyloid and tau protein which is constituents of senile plaques and neurofibrillary tangles respectively. Methyline blue (Rember) is known to interfere with tau aggregation is investiaged for its efficacy [Wischik *et al*., 1996]. The results of Phase II trail of methyline blue showed its benefits in the subset of participants [Gura, 2008]. Phase III trails are underway to know its benefits, efficacy and safety of methyline blue.

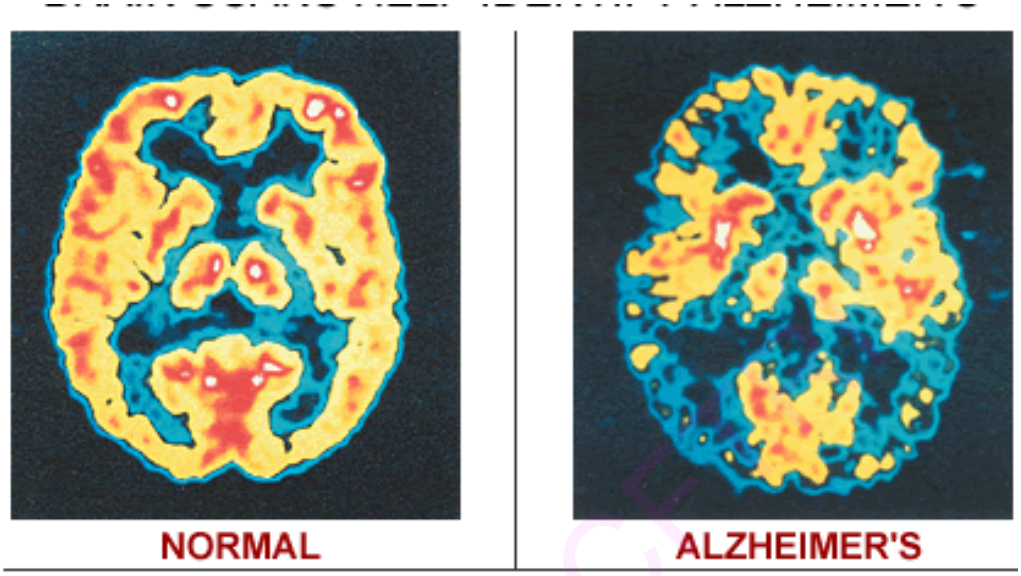


Fig 1.4: PET scan images of normal and Alzheimer's disease: In case of AD brain reduced glucose metabolism shown as reduction red colored areas compared to control **(**Images are courtesy of Alzheimer's disease Education and Referral Centre, National Institute on Aging).

1.3. Oxidative stress and AD

1.3.1. Mitochondria and oxidative stress

Oxidative stress is the result of imbalance between antioxidants and free radicals in AD [Markesbery, 1997]. Oxidative stress is one of the earliest events in AD [Smith *et al*., 2000]. Oxidative stress associated with mitochondria is further triggered by neurodegenerative process, metabolic demand and age. Further ageing is the risk factor for neurodegeneration and AD. The products of oxidative damage are known to be associated with Aβ deposits and NFT. Mitochondria are known as a powerhouse of a cell as it generates energy. Mitochondria are an important source of oxidative stress as it generates free radicals. Free radicals are formed due to incomplete reduction of oxygen during the respiration process in mitochondria. Approximately 1% of molecular oxygen, which is entering respiratory chain, is incompletely reduced leading to the formation of superoxide [Boveris and Chance 1973].The mitochondria

contribute reactive oxygen species in the form of $O^{\bullet-}_2$ and H_2O_2. The evidences suggest that mitochondrial dysfunction in AD is attributed to abnormalities in mitochondrial genome [Corral-Debrinski *et al*., 1992; Davis *et al*., 1997] or deficiencies in key mitochondrial enzymes [Sorbi *et al*., 1983; Sheu *et al*., 1985; Blass *et al*., 1990; Parker *et al*., 1990]. The conversion of toxic $O^{\bullet-}_2$ is mediated by mitochondrial superoxide dismutase-1. This enzyme is found to be defective in AD. The reduced function of mitochondria in AD is attributed to accumulation of APP in mitochondria [Ananthatheerthavarad *et al*., 2003; Devi *et al*., 2006; Keil *et al*., 2004; Park *et al*., 2006]. Additionally, APP695 accumulation in human cortical neuronal cell line was linked with reduced membrane potential decreased ATP generation and reduced cytochrome c activity [Ananthatheerthavarad *et al*., 2003]. Also, amyloid is found in mitochondria of human AD brain [Lustbader *et al*., 2004; Devi *et al*., 2006] and in AD transgenic mice [Caspersen *et al*., 2005; Manczak *et al*., 2006; Crouch *et al*., 2005]. There is a direct link between Aβ and mitochondrial dysfunction. Aβ interacts with Aβ binding alcohol dehydrogenase (ABAD) of AD patients and in transgenic mice and inhibits its activity [Lustbader *et al*., 2004]. X-ray crystallographic studies showed that Aβ compete with nicotinamide adenine dinucleotide for active site in ABAD which further leads to oxidative stress [Lustbader *et al*., 2004]. Both *in vitro* and *in vivo* studies revealed that Aβ in the mitochondria is associated with increased levels of hydrogen peroxide, decreased cytochrome c activity and higher levels of carbonylated proteins [Manczak *et al*., 2006]. The ABAD has significant physiological role in mitochondria and mutational inactivation results in lethal phenotype in Drosophila [Torroja *et al*., 1998]. ABAD is up-regulated in affected neurons in AD and co-expression of APP with mutant amyloid APP exacerbates Aβ induced cellular oxidant stress and cell death [Yan *et al*., 1997; Yan *et al*., 1999].These data suggests that there is a interaction between Aβ and ABAD which resulted in mitochondrial dysfunction. The transgenic mice over-expressing ABAD in presence of high Aβ concentration results in elevated neuronal oxidative stress and impaired memory (Lustbader *et al*., 2004). Free radicals generated by as result of mitochondrial dyfunction attack macromolecules such as proteins, DNA, RNA and lipids [Aksenov *et al*., 2001; Ding *et al*., 2006; Markesbery *et al*., 2005; Wang *et al*., 2006]. Additionally, Aβ accumulation in mitochondria leads

to decreased activities of complex III and complex IV and it is associated with reduced oxygen consumption [Caspersen *et al*., 2005]. Inhibiting the complex IV and α□-ketoglutarate dehydrogenase results in increased reactive oxygen species production [Lin and Beal, 2006]. **Oxidative stress in Down's syndrome (DS) and AD:** Downs syndrome is neurogenetic disorder characterized by abnormalities in children leading mental retardation and premature aging. Children suffering from Down's syndrome is known to have congenital malformations in addition to leukemia's, cataracts and growth retardation [Roizen and Patterson, 2003]. Additionally, immune disorders such as Celiac disease, thyroid dysfunction and diabetes mellitus has been found to be associated with DS [Zana *et al*., 2006]. Genetically, DS is characterized by trisomy of short arm human chromosome 21. This means the genes that are present in the human chromosome 21 has double copy of the genes than in normal persons. The genes that are present in the human chromosome 21 which are associated with DS are APP and cytoplasmic enzyme cu2+ /Zn2+ super oxide dismutase [SOD1] coding genes. Higher expression of these two genes is correlated with increased oxidative stress in DS [Schuchmann and Heinemann, 2000]. The gene dosage effect infers that there is a alteration in the homeostasis of specific individual gene or small group of genes responsible for onset of Down syndrome [Schuchmann and Heinemann, 2000]. This indicates that an extra copy of genes that matters, rather than the consecutive expression of genes [korenberg *et al*., 1990; Reeves *et al*., 2001]. Evidence suggests that Down syndrome is a risk factor for AD [Bush and Beal 2004]. Oxidative stress may play a common role both in DS and AD. SOD1 plays significant role in converting toxic oxygen radical ($O^{\bullet-}_2$) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). Hydrogen peroxide will be further converted to hydroxyl radical and water by catalase and glutathione peroxidase in the barin [Zana *et al*., 2006]. All these enzymes will be in homeostatic condition in normal persons, while in DS there is double dose of SOD1 which creates imbalance among SOD1, catalase and glutathione peroxidase. This leads to over accumulation of hydrogen peroxide which in turn leads to oxidative stress. The over-expression of APP in DS may be a protective toil in DS. APP is known to protect the neurons against oxidative stress induced deleterious affects [Isacson *et al*., 2002].

1.3.2. Why brain is susceptible for oxidative stress?

Oxidative stress is a process leading to the production of reactive oxygen species, where it causes molecular damage can leading to altered biological functions [Rottkamp *et al*., 2000]. The brain is rich in unsaturated fatty acids, thus it is prone to oxidative stress. The degree of unsaturation of fatty acids in the brain is more prone to oxidative stress than the number of fatty acids [Barja, 2004]. The unsaturated fatty acids in the macromolecules such as mitochondria and nucleus are more sensitive to free radical damage [Barja, 2004]. So, this indicated that decrease in degree of unsaturation (number of double bonds) decreases oxidative damage by free radicals. The main source of free radical generation in AD is mitochondria. Both complexes I and II of mitochondria leak radicals, which contribute to radical pool. The decrease in the activity of cytochrome oxidase is well documented in AD [Parker *et al*., 1994; Cardoso *et al*., 2004]. Other antioxidant enzymes which are decreased their activity in AD include pyruvate dehydrogenase, glutamine synthetase, creatine kinase and aconitase [Sorbi *et al*., 1983; Kish, 1997; Gibson *et al*., 2000]. Redox-active metals such as Fe and Cu are involved in the production of reactive oxygen species in the brain and the levels of Fe and Cu are high in brain tissues. Iron (Fe) and copper (Cu) are implicated in the formation of oxygen free radicals and damage tissue in AD brain [Smith *et al*., 1997]. In particular, it is reported to be important cause of oxidative stress in AD [Lovell *et al*., 1998] Fe gets accumulated in neurofibrillary tangles (NFTs) as well as in Aβ deposits [Good *et al*., 1992]. Aluminium (Al) is also found to accumulate in NFT containing neurons [Muma and Singer, 1996; Garruto *et al*., 1984] and found to stimulate the Fe–induced lipid peroxidation [Oteiza, 1994]. There are reports on increased levels of Zn (II), Fe (III) and Cu (II) in the neuropil and senile plaques in the AD brain [Castellani *et al*., 1999]. Al is also known to aggravate the free radical damage already initiated by Fe [Rao *et al*., 1999]. It increases the production of free radicals primarily through the Fenton reaction between hydrogen peroxide and abridged transition metals [usually iron(II) or copper (I)] [Fenton,1984].

$$H_2O_2 + Fe^{2+} + O^{2}* + Fe^{3+}$$

The responses to oxidative stress in AD include activation of stress activated protiein kinase [SAPK]. SAPK is the main mediator of defense mechanism against the oxidative stress in AD [Moreira *et al*., 2005]. The nuclear localization of JNK/ SAPK in neurons confirmed that these pathways have definite role in mediating oxidative stress.

1.4.Genes and AD: Alzheimer's disease can be divided in to familial and sporadic forms. In the familial form of AD, more than one person in the same family is affected with AD. In case of sporadic form, only one person in the family is affected with AD. Only 20% of the AD is familial form and remaining is sporadic form [Bird, 2007]. The involvement of three genes in the cause of familial AD is well established (Goedert and Spillantini, 2006). These genes are known contribute significantly to the onset of familial AD. These three genes include APP gene, PS1 gene and PS2 gene. Mutations in APP accounts for 10-15% of familial Alzheimer's disease [Bird, 2007]. The PSEN (presenilin) gene codes for proteins that cleave APP. PS1 codes for β-secretase, which cleaves at N-terminal of Aβ in APP and PS1 is located in human chromosome 14. PS2 codes for complex protein called γ□-secretase which cleaves at C-terminal of Aβ in APP and it is located in human chromosome 1. The mutations in PS1 accounts for 30-70% of the familial Alzheimer's disease, while mutations in PS1 accounts for approximately 5% of familial Alzheimer's disease. Inheritance of APP, PS1 and PS2 follow an autosomal dominant pattern and results in development of familial Alzheimer's disease [Bird, 2007]. The offspring of familial Alzheimer's disease affected parent have 50% chance of inheriting and developing to familial Alzheimer's disease. But all the familial Alzheimer's disease patients did not show mutations in APP, PS1 and PS2. The other genes that are considered as risk factors for AD includes, apolipo-protein E (ApoE ε 4 variant) [Porier *et al*. 1996], α-macroglobulin [Blacker *et al*., 1998], K-variant of butyryl-cholinesterase [Lehman *et al*., 1997]. Uhrig *et al*. [2009] reported new genes. NEUROG2 and KIAA0125 which are responsive to Aβ. They demonstrated the expression levels of NEUROG2 and KIAA0125. Their expression levels are inversely proportional and are altered by ratio of Aβ42/ Aβ40. The increase in Aβ42/ Aβ40 ratio increases the expression levels of NEUROG2 and down regulates KIAA0125. While decrease in Aβ42/ Aβ40 decreases NEUROG2 and up regulates KIAA0125. They speculated that KIAA0125 could be

involved in neurogenesis while NEUROG2 is involved in the development of neural processes. This information will suggests that Aβ regulates other genes and there may be many more to explore.

1.5.Diet and AD

1.5.1.Caloric restriction (CR) and AD

The first lesson in moderation comes from calorie restriction. Energy requirements decline progressively after early adulthood, because of cessation of growth and a decrease in basal metabolic rate and physical activity. The average energy requirement for an adult is 25 kcal/kg/day whereas for children it is the sum of 100 kcal/kg/day for the first 10 kg, 50 kcal/kg/day for the second 10 kg and 20 kcal/kg/day beyond 20 kg [Gillenwater *et al*., 1991]. It has been shown that calorie allowances will be reduced by 5% per decade between 35 and 55, 8% per decade between 55 and 75 and a further 10% beyond 75 years of age although this may vary considerably based on physical activity and other characteristics [Rovio *et al*., 2005]. Dietary excess is known to influence the onset and progress of age related diseases like diabetes, obesity and vascular diseases and actually has been shown to reduce life-spans [Sun and Alkon, 2006; Leibson *et al*., 1997; Peila *et al*., 2002]. CR known to reduce the production of reactive oxygen species in animals by modulating neuroinflammation and oxidative stress [Lee *et al*., 2000; Morgan *et al*., 1999], and CR is reported to have neuroprotective effects in young rodent models of neurodegenerative disease [Bruce- Keller *et al*., 1999; Mattson, 2003]. Furthermore, CR activates intercellular neurotrophic signaling mechanisms and thus provides neuroprotection [Mattson, 2000]. Recent studies strongly suggest a link between diets and AD leading to the notion that CR may delay or prevent AD [Luchsinger *et al*., 2002; Engelhart *et al*., 2002; Gustafson *et al*., 2002; Grant, 2004; Mattson *et al*., 2002]. There is still a debate about whether CR works to increase life span, and by extension, other benefits in humans although the bad effects of dietary excess remain unchallenged [Lee *et al*., 2000]. Nevertheless, data from population based studies suggest that a lower calorie intake leads to a lowered risk of AD and PD [Luchsinger *et al*., 2002; Bruce- Keller *et al*., 1999; Logroscino *et al*.,1996]. CR is known to

induce neuroprotective molecules that have a role in resistance of neurons to oxidative, metabolic, excitotoxic and apoptotic insults [Lee *et al*., 2000; Duan *et al*., 2001; Yu and Mattson, 1999; Lee *et al*., 2002]. CR is known to induce expression of several different neurotrophic factors like brain derived neurotrophic factor (BDNF) in brain cells [Lee *et al*., 2000]. Rats maintained on CR show increased levels of brain-derived neurotrophic factor in neurons in cerebral cortex, hippocampus, and striatum [Lee *et al*., 2002; Duan *et al*., 2003]. BDNF has been reported to have role in enhancing memory and learning, and protects neurons against oxidative and metabolic insults and is also known to stimulate neurogenesis [Lee *et al*., 2002; Duan *et al*., 2003]. Studies also show reduced levels of brain-derived neurotrophic factors in patients with AD and PD [Hock *et al*., 2000; Howells *et al*., 2000]. There is a substantial amount of data accumulating in favor of increase in life span by stimulating silent mating type information regulation-2. The silent mating type information regulation-2 is required in some species for enhanced life span [Hunt *et al*., 2006]. CR has also been demonstrated to be capable of reducing the amyloid and NFT lesion load of animal models suggesting that it may directly affect the pathogenesis of AD [Qin *et al*., 2006; Halagappa *et al*., 2007]. However, there is a need for more data generation on calorie restriction and its benefits to the brain. The mechanism by which CR modulates AD is not clear, however, it is postulated that CR increases synaptic plasticity, anti-inflammatory mechanisms and inducing neuroprotective factors [Gillette-Guyonnet and Vellas, 2008]. CR was shown to protect the age-related loss of neurons in AD [Wu *et al*., 2008]. They also observed that CR could reduce the enlargement of ventricles, caspase activation and astrogliosis [Wu *et al*., 2008]. From the above findings it indicated that CR could increase neurogenesis [Levenson and Rich, 2007] but, before we come to consensus, we should also analyze and quantify the long-term effects of CR in adults [Gillette-Guyonnet and Vellas, 2008].

However, before rushing to advocate CR as a treatment paradigm it is important to recognize that the treatment may be too late and even be counterproductive. AD patients commonly develop abnormal eating behaviors [Blandford *et al*., 1998] that include anorexia nervosa and bulimia nervosa [Riviere *et al*., 2002]. Studies indicate that dietary intake of nutrients is poor in older adults who convert to early stage AD as

compared to their cognitively intact counterparts [Shatenstein *et al*., 2007]. Indeed weight loss may be an early indicator of AD prior to dementia [Luchsinger and Gustafson, 2009].

It is important to emphasize that little is known about the factors correlated with eating difficulties in AD. Riviere and colleagues [Riviere *et al*., 2002] conducted a one-year investigation to understand the factors responsible for adverse eating behavior among AD patients living at home with caregivers. This study involved 224 patients and their caregivers. Eating difficulties were assessed using the eating "Dependency Scale and Averse Eating Behavior Inventory". The study found two significant associations which include: i) eating difficulties and age of the caregivers and ii) severity of the disease and psychological function of the patients [Riviere *et al*., 2002]. They analyzed the data using the regression analysis and found positive correlation between adverse eating behavior and initial caregivers burden. There was, however, an inverse correlation between memory impairment and adverse eating behaviors. Thus, both cognitive impairment and family stress can help in predicting that AD patients living at home develop adverse eating behavior.

1.5.2.Lipids and AD

Lipids have two major functions in the cell: they are reservoirs of chemical energy stored as fat and they are the structural components of cell membranes. Lipids also act as signaling molecules through steroid hormones and eicosanoids. Lipid metabolism plays a key role in AD through the ApoE-ε4 allele, one of the genetic risk factors for AD [Basset and Montine, 2003; Puglielli *et al*., 2003; Jarvik *et al*., 1995; Pentaceska *et al*., 2003]. The transport of lipid in blood, brain and cerebrospinal fluid is modulated by ApoE [Jarvik *et al*., 1995; Marshall *et al*., 1996]. There is also an important association between dietary factors and ApoE polymorphisms, which gives a clue as to why we should consider dietary fat uptake patterns in different populations [Farrer *et al*., 1997]. There is a hypothesis that one dose of e4 allele in the brain increases AD risk by 2-3 times, two doses of ε4 allele provides a 12-15 times higher risk [Farrer *et al*., 2003; Petot *et al*., 2003].

An interesting study has been conducted to assess the risk factors throughout adult life based on a life history questionnaire; it includes medical, occupation, activity level, education, smoking and dietary habit questions [Lopez-Miranda *et al*., 1994]. The investigations have obtained lifestyles pattern for three age groups: 20-39, 40-59 and 60 + or 5 years prior to AD diagnosis for cases. They indicated that healthy controls with the ApoE ε4 allele consumed less total and saturated fat between the ages of 20 and 60 years than those without the ε4 allele [Lopez-Miranda *et al*., 1994]. The consumption of higher fat through diet during mid-life may therefore reduce the risk for AD than for those without the e4 allele. This may indicate a protective effect of the ApoE ε2 allele and ε3 allele due to the modulating effects of ApoE on LDL cholesterol level [Campos *et al*., 2001; Hu and Willet, 2007]. The intake of non-hydrogenated unsaturated fats, low intake of hydrogenated and saturated fats and high intake of n-3 polyunsaturated fatty acids from fish or vegetable sources may lower the risk of vascular dementia as well [Lim *et al*., 2005].

A relationship between reduced risk of AD and a diet rich in docosahexaenoic acid (DHA) and omega-3 essential polyunsaturated fatty acid was reported [Calon *et al*., 2004]. Furthermore, a possible role for DHA in preventing lipid peroxidation and in reducing the accumulation of Aβ in the cortico-hippocampal region in the mouse model was hypothesized [Kitjka *et al*., 2002]. It has also been shown that DHA plays a role in the expression of signal transduction molecules [Puskas *et al*., 2003]. Additionally, DHA is known to stimulate the expression of transthyretin, a protein involved in the transport of thyroxin. Transthyretin has an affinity for Aβ and possibly stimulates the clearance mechanism of Aβ [Schroeder *et al*., 1995]. These observations support the need for further research to understand the therapeutic potential of DHA in AD.

Cholesterol is present in specialized membranes of myelin and in the membranes of neuronal and glial cells present in the brain (approximately 25% of the total cholesterol of the human body) [Porter *et al*., 1996; Yeagle, 1991; Mitchell *et al*., 1990; Brown and Golgstein, 1997; Rouser *et al*., 1972]. Alterations in the metabolism of cholesterol are reported to be associated with age [Sakakihara and Volpe, 1985; Roth *et al*., 1995; Igbavboa *et al*., 1996; Lutjohann *et al*., 1996; Reiss *et al*., 2004;

Mason *et al*., 1992] and have been shown to play a role in the pathogenesis of AD [Jarvik *et al*., 1994; Koudinov and Koudinov, 2001; Puglielli *et al*., 2001]. Recent reports show a link between cholesterol and AβPP processing pathways [Bodovitz and klein, 1996; Nuan and Small, 2002]. An increase in dietary cholesterol levels increase secreted AβPP derivatives, namely sAβPPα and sAβPPβ in mouse brain and modulate the levels of major secreted Aβ forms $A\beta_{40}$ and $A\beta_{42}$ [Bodovitz and klein, 1996] Recent studies suggest that β- and γ-secretase may be regulated by isoprenoid that are synthesized in the cholesterol biosynthesis pathway in addition to cholesterol [Cole *et al*., 2005; Refolo *et al*., 2000].

Transgenic mouse models over expressing human AβPP, and maintained on a diet rich in saturated fats and cholesterol, have shown an increased accumulation of Aβ alone or in combination with other AD-related proteins [Granholm *et al*., 2008]. Additionally, the dietary fats (saturated fat, hydrogenated fat and cholesterol) are reported to be involved in the impairment of memory and hippocampal pathology in the rat brain [Patil and Chan, 2005]. Conversely, inhibition of cholesterol synthesis reduces amyloid load.

Though the etiology of AD is still elusive, reports have strongly linked the role of cholesterol and ApoE in AβPP processing. Elevated levels of saturated fatty acids in AD and its role in hyperphosphorylation of tau were reported [Panza *et al*., 2004]. To examine free fatty acid induced hyperphosphorylation of tau, they studied primary rat cortical neurons in untreated (control) and treated neurons with 0.2mM of either palmitic or stearic acids for 24h. The findings indicated that astroglia mediated oxidative stress is found to be involved in free fatty acid-induced hyperphosphorylation of tau in primary neurons. Further, it was reported that saturated fatty acids might induce aggregation of tau, as well as Aβ [Panza *et al*., 2004]. A study indicated that higher intake of saturated fatty acids may be a risk factor for AD [Reaven *et al*., 1994]. However, the question is whether or not a higher intake of polyunsaturated fatty acids and monounsaturated fatty acids will reduce the risk for AD. A word of caution is the quantity of uptake of polyunsaturated fatty acids and monounsaturated fatty acids, and their link to atherogenesis. As an example, a

high intake of linoleic acid, which is n-3 polyunsaturated fatty acids, may increase the susceptibility of LDL cholesterol to oxidation leading to atherogenesis [Arendash *et al*., 2007]. The recent report suggests that diet rich in omega-3 fattyacids or use of fish oil supplements (DHA and EPA), did not protect against AD. But the dietary fish may contain nutrients, other than DHA and EPA, that may provide some protection against AD [Pappolla *et al*., 2003].

In conclusion, lipids have a complex relationship with AD. Some appear to be protective whereas others such as cholesterol appear to be harmful. The relationship is however even more complex with some of the effects of cholesterol attributable to intermediates in cholesterol biosynthesis such as isoprenoids. Interestingly, the relationship between AD and cholesterol epidemiology is complex with almost no difference between normal controls and AD. However, high levels of cholesterol in middle age has been linked to higher risk for AD in later life [Mielke *et al*., 2005]. Further complicating the picture is the finding that high cholesterol late in life is actually linked to a reduction in AD risk [Sambamurti *et al*., 2005] suggesting that AD is likely linked to a rapid loss of cholesterol with age [Rottkamp *et al*., 2000].

1.5.3.Metal chelators in AD

The brain is rich in unsaturated fatty acids, and thus it is prone to oxidative stress. Redox-active metals such as Fe and Cu are involved in the production of reactive oxygen species in the brain, and the levels of Fe and Cu are high in brain tissues. Oxidative stress is a process leading to the production of reactive oxygen species, where it causes molecular damage leading to altered biological functions [Smith *et al*., 1997]. Moreover, Fe and Cu are implicated in the formation of oxygen free radicals and damage tissue in AD brain [Good *et al*., 1992]. Fe accumulates in neurofibrillary tangles (NFTs) as well as in Aβ deposits [Muma and Singer, 1996]. Aluminum (Al) also accumulates in NFT-containing neurons [Garruto *et al*., 1984; Oteiza, 1994] and is found to stimulate Fe-induced lipid peroxidation [Castellani *et al*., 1999]. There are reports on increased levels of Zn (II), Fe (III), and Cu (II) in the neuropil and senile

plaques in the AD brain [Rao *et al*., 1999]. Al is also known to aggravate the free radical damage already initiated by Fe [Sparks and Sheurs, 2003].

Interestingly, metals such as Cu (II) appear to facilitate the cholesterol-mediated increase in amyloid pathology [Lahiri *et al*., 2008]. Early exposure to Pb is also implicated in increased amyloidosis by increasing APP expression [Hedge *et al*., 2009]. Several dietary spices are known to act as metal chelators and may therefore be protective against dementia [Butterfield *et al*., 2002].

1.5.4.Vitamin E and AD

The body's defensive system against oxidative stress includes molecules called antioxidants that are also known as free radical scavengers. There is still a debate regarding the role of reactive oxygen species related to neuronal damage. Considering that oxidative stress can be a primary event, the role of dietary antioxidants in combating oxidative stress in AD is discussed below. Additionally, the dysregulations of metabolic pathways in the aged brain will lead to reduced synthesis of defense molecules to combat oxidative stress. The major questions a still to be understood are: What are the regulatory molecules of the metabolic pathways in aged brain that are susceptible to oxidative damage? What are the homeostatic mechanisms make neurons resistant to oxidative damage?

Studies have reported the role of antioxidants in lowering the risk of stroke and AD [Ascherio, 2000; Hirvonen *et al*., 2000; Zandi *et al*., 2004; Kedar, 2003]. The change in the concentration of antioxidants in neurodegeneration may be a primary or secondary event in relation to dietary intake [Kedar, 2003]. Studies have established a relationship between the plasma concentration of antioxidants and cognition [Luchsinger *et al*., 2003; Cavallini *et al*., 1978]. Studies indicate that the various antioxidant supplements could be effective in reducing oxidative stress [Fusco *et al*., 2007; Joesph *et al*., 1998].

Notably, the level of vitamin E in plasma of AD patients is 18.65±3.62 mmol/L compared to age-matched controls 30.03±12.03mmol/L [Morris *et al*., 2005; Peterson *et al*., 2005]. It has been shown that long term feeding of rats (from 6 to 15 months of

age; F344 rats) with a supplemented AIN–93 diet (strawberry or spinach extract (1-2 % of the diet) or vitamin E (500 IU), have protected against age-related changes in cognitive functions. Furthermore, the supplemented diet could prevent the onset of age related deficits in several indices, including cognitive behavior and performance with the Morris water maze [Morris *et al*., 2005; Peterson *et al*., 2005]. However, Petersen and colleagues [Young and Greenwood, 2001] indicated that vitamin E had no beneficial effect in patients with mild cognitive impairment [Young and Greenwood, 2001]. In a double blind study [Young and Greenwood, 2001], subjects with mild cognitive impairment were given 2000 IU of vitamin E daily, 10 mg donepezil daily, or a placebo for three years. The overall rate of progression from mild cognitive impairment to full clinical AD was 16% per year, and, importantly, there was no difference between subjects on the placebo and subjects who received vitamin E over a period of three years. These studies disagree on the validity of vitamin supplements to AD patients and, thus, more research is essential to understand further.

Researchers across the globe are interested in elucidating the protective potential of vitamin E and vitamin C against AD [Sumien *et al*., 2003]. In another major study, AD patients were given 2,000 IU of vitamin E per day, a dose that exceeds the recommended daily allowance of vitamin E. They found that a higher dose of vitamin E is able to delay the admission to a nursing home by six months, compared to those taking a placebo [Sumien *et al*., 2003]. The question is to understand the quantity of vitamins required in reducing the risk of AD and safety of vitamin E at higher concentrations. Further, the dietary supplements of vitamin E fail to provide better results compared to dietary intake of vitamin E [Kontush and Schekatolina, 2004]. If the supplements are less beneficial than dietary vitamin E, what is the main reason for such a difference? The probable reason might be the composition of the diet, which has a cumulative and synergistic effect in the vitamin bioavailability. The apparent protection provided by dietary vitamins E and C could be by synergy due to other substances in fruits and vegetables, such as flavonoids, which have both anti-inflammatory and antioxidant properties [Seshadri and Wolf, 2003].

The clinician should diagnose the plasma concentration of antioxidants at a particular point of AD grading and make a decision on recommending the use of vitamin E supplements.

1.5.5.Vitamins and homocysteine interrelations

Hyperhomocysteine levels induce neurologic abnormalities such as cerebral atrophy, and seizures, etc. [He *et al*., 2004]. A deficiency of vitamins is found to elevate the concentration of homocysteine, which is implicated in vascular mechanisms leading to AD [He *et al*., 2004; Leboef, 2003]. Vitamins like folate, B6, and B12 have been involved in the biosynthesis of amino acids, which contain sulphur, methionine, and cystein [Clarke *et al*., 1998]. Folate and vitamin B12 are involved in biosynthesis of methionine from its precursor homocysteine, whereas, B6 has a role to play in the conversion of homocysteine to cysteine. The levels of homocysteine in the blood are elevated with ageing and age is one of the risk factor for AD [Mattson, 2000]. The factors like folic acid and caloric intake are also known to modulate the plasma homocysteine [Mattson, 2000]. There is an inverse relationship between plasma folic acid and homocysteine, and dietary folic acid is found to lower homocysteine levels.

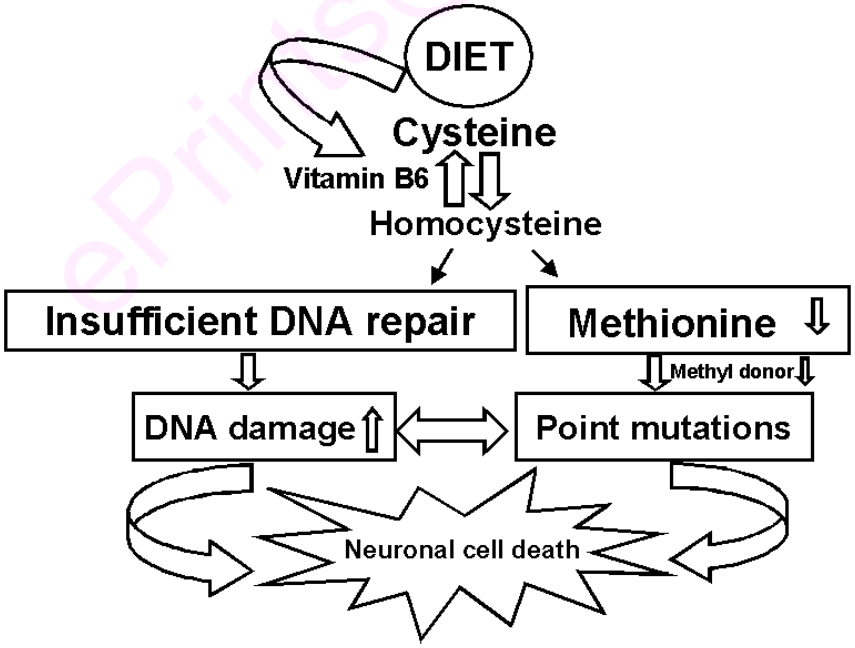


Figure 1.5: Insights into the relationship between vitamins and homocysteine relative to AD. Increased levels of homocysteine in AD patients are a known risk factor.

Higher homocysteine levels causes insufficient DNA repair and point mutations, which leads to accumulation of DNA damage and cell death. The reduction in the levels of homocysteine can be brought about by increasing cysteine levels in the body through diet. Vitamin B6 acts as a cofactor in the transformation of homocysteine to cysteine.

Additionally, calorie restriction is also found to decrease homocysteine levels, but the magnitude of the effect is moderate [KrumanII *et al*., 2002]. There are higher homocysteine levels in the patients with deficient in enzyme cystathione β synthase, which is involved in catabolism of cystein. A study has shown that in mice fed in a diet with reduced levels of folic acid, there is hipppocampal pyramidal neuronal degeneration [Baydas *et al*., 2003]. This may be because of the elevated levels of homocysteine in mice due to low folic acid. Also, administration of homocysteine into the brain is found to enhance neuronal degeneration [Kruman II *et al*., 2000], and elevated levels of homocysteine induce accumulation of DNA damage in neurons [Kruman II *et al*., 2002; Baydas *et al*., 2003; Fuso *et al*., 2005]. This may be because increased homocysteine induces a deficiency of methyl donors, which has implication on uracil misincorporation and oxidative damage to DNA bases [136 Baydas *et al*., 2003, Fuso *et al*., 2005]. A link between levels of homocysteine and gene expression was also reported and increased homocysteine leads to decrease in the levels of methyl donors, causing hypomethylation of PS1 promoter [Fuso *et al*., 2008]. It is likely to alter the gene expression as gene silencing is mediated by the methylation of the promoter [Fuso *et al*., 2008]. Fig 1.5 represents the details of homocysteine and neuron cell death. A number of studies suggest that homocysteine can increase amyloid load in transgenic mice [Pacheco-Quinto *et al*., 2006]. All the above events provide insights into the relationship between dietary intake of vitamins and homocysteine and their effect in neuronal cell death. We therefore hypothesize a relationship between vitamins, homocysteine and neuronal dysfunction. The increased levels of homocysteine in AD patients are a risk factor. Higher homocysteine levels cause insufficient DNA repair and point mutations, which leads to the accumulation of DNA damage and cell death. A reduction in the levels of homocysteine can be brought about by increasing cysteine levels in the body through the use of vitamin B6, which acts as a cofactor in the transformation of homocysteine to cysteine or by recycling it back to methionine using vitamin B12 and folate. Indeed, a deficiency in

vitamin B12 leads to a form off dementia that resembled AD in clinical presentation and can be reversed by diet.

1.5.6.Dietary polyphenols and AD

Polyphenols are natural substances that are present in plants, and their quantities vary in leaves, flowers, vegetables, and fruits. Considerable amounts of these compounds, moreover, are present in olive oil and red wine [Jang and Surh, 2003]. Among the polyphenols, flavonoids occupy the largest group [Butterfield *et al*., 2002]. The major component of green tea flavanoids, EGCG, for instance, has recently been shown to have neuroprotective functions such as antioxidation, iron chelation, and anti-inflammation [Mandel *et al*., 2007]. Specifically, the abundant phenolic hydroxyl groups on the aromatic ring confers the antioxidant activity, and the 3-OH group is essential for iron chelating activity of these compounds [Van Acker *et al*., 1996]. Similarly, the Mega Natural grape seed polyphenolic extract (GSPE), derived from grape seed, significantly inhibits oligomerization of Aβ and restores the cognitive deterioration [Wang *et al*., 2008]. These trends ultimately provide a clue that polyphenols can be good intervention molecule for neurodegeneration.

Tea polyphenols have been found to be potent scavengers of free radicals [Salah *et al*., 1995; Morel *et al*., 1995]. EGCG contains three heterocyclic rings, A, B, and C, and the free radical scavenging property of EGCG is attributed to the presence of trihydroxyl group on the B ring and the gallate moiety at the 3' position in the C ring. EGCG is also known to chelate transition metal ions like iron and copper [Mandel *et al*., 2007]. There are two sites where metal ions bind to the flavonoid molecule: 1) o-diphenolic group in the 3',4'-dihydroxy positions in the B ring, and 2) keto structure 4-keto, 3-hydroxy in the C ring of flavonols [Van Acker *et al*., 1996; Thompson *et al*., 1976]. Further, EGCG is found to have role in elevating the activity of two major antioxidant enzymes, superoxide dismutase, and catalase in the mouse striatum [Levites *et al*., 2002]. Finally EGCG treatment has been reported to modulate AβPP processing to Aβ [Rezai-Zadeh *et al*., 2005]. The mechanisms regarding oxidative stress and its augumentation by EGCG are highlighted in fig 1.6.

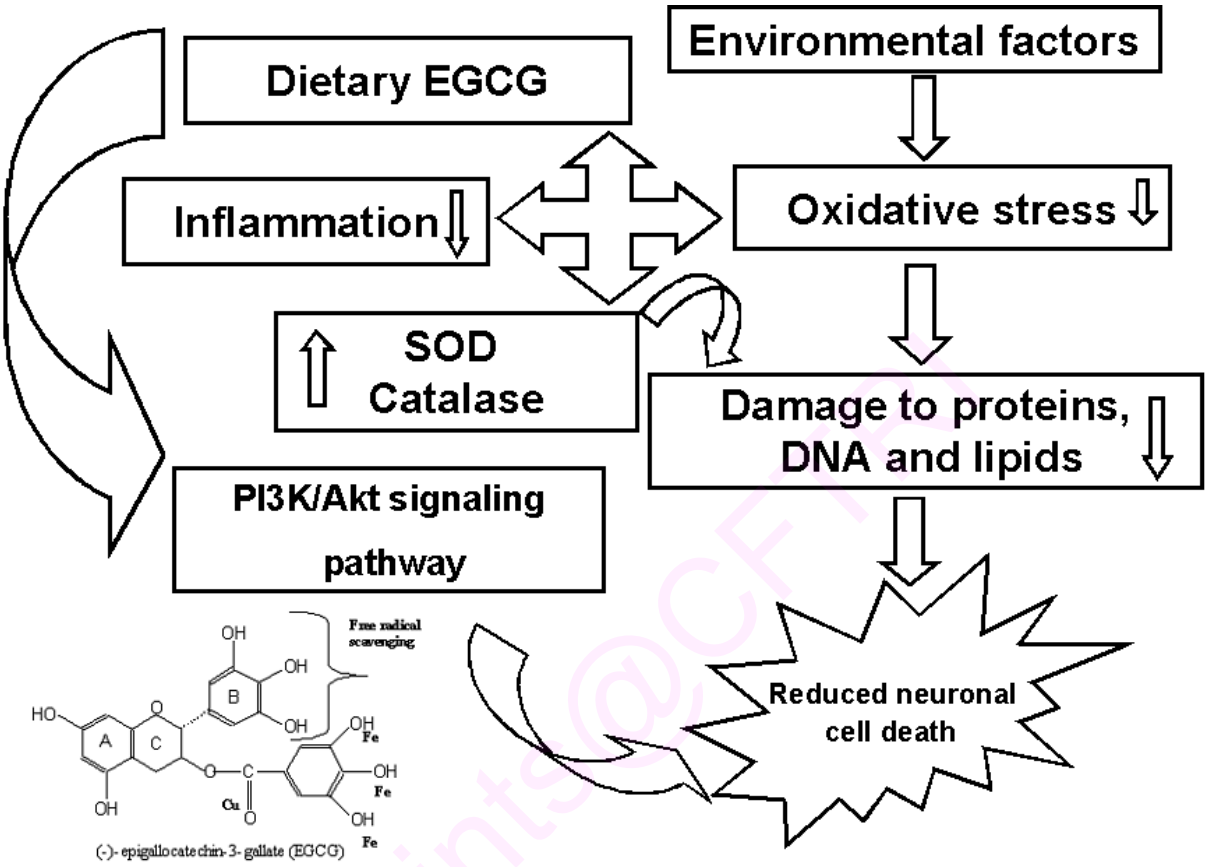


Figure 1.6: EGCG's mode of action. Environmental factors such as trace metals cause oxidative stress, which then induce protein, lipid, and DNA damage. This leads to increased susceptibility of neurons to stress and cell death. EGCG is known to chelate transition metals like Fe and Cu, which reduce oxidative stress. It also acts as an anti-inflammatory molecule. EGCG induces the PI3K/Akt-signaling pathway, which is important for neuronal cell survival. The figure also indicates the functional groups in EGCG and their activities in transitional metal and free radical chelation.

1.5.7.Wine and AD

A number of researchers have explored the relationship between alcoholic beverages and AD [Ruitenberg *et al*., 2002; Mukamal *et al*., 2003; Galanis *et al*.,

2003; Truelsen *et al*., 2002]. Studies have shown that frequent alcohol uptake in rats can result in mitochondrial dysfunction in neurons leading to neurodegeneration [Jaatinen *et al*., 2003]. In contrast, it is reported that moderate alcohol uptake is related to a lower risk of clinical stroke [Sacco *et al*., 1999]. Thus, alcohol may have paradoxical and competing effects in the brain; it lowers the risk of cerebrovascular disease and also likely acts as a neurotoxin. Researchers have shown a relationship between alcoholic drinks and AD [Hooijmans and Kiliaan, 2008; Ruitenberg *et al*., 2002; Mukamal *et al*., 2003; Galanis *et al*., 2000; Truelsen *et al*., 2002]. A study of people aged 65 years and older showed that alcohol consumption of one to six drinks a week, regardless of the type of beverage used, lowered the risk of AD compared to abstainers [Mukamal *et al*., 2003]. In another study, people who consumed three servings of alcohol a day had a low risk of AD compared to those who were never exposed to alcohol [Ruitenberg *et al*., 2002]. An interesting study involving individuals aged 65 years and older found that monthly or weekly intake of wine, but not other alcoholic drinks, was associated with a lower risk of dementia including AD [Truelsen *et al*., 2002; Lindsay *et al*., 2002]. Most of the results obtained in the above studies were not statistically significant, given the small number of elderly people participating in the studies.

However, several epidemiological studies have shown that moderate wine consumption reduces the risk of developing AD [Hooijmans and Kiliaan, 2008; Truelsen *et al*., 2002; Lindsay *et al*., 2002; Orgogozo *et al*., 1997]. Wine contains antioxidants such as resveratrol, a flavonoid, which is not present in beer or other spirits. Resveratrol occurs in abundance in grapes and red wine [Jang and Surh, 2003; Savaskan *et al*., 2003]. The beneficial effect of wine consumption on the neurodegenerative process is therefore attributed to resveratrol [Jang and Surh, 2003; Savaskan *et al*., 2003; Han *et al*., 2004; Marambaud *et al*., 2005], and resveratrol was reported to reduce Aβ production in cell line HEK293 expressing wild type or Swedish- mutant APP695 [Marambaud *et al*., 2005]. Additionally, studies indicate the involvement of resveratrol in proteosome clearance of Aβ; decreases in its presence reduce Aβ clearance and reduce toxicity in AD brains. The proteosome is an ubiquitin activated protein quality control system that enzymatically labels, transports, and

finally degrades misprocessed and misfolded protein [deVrij *et al*., 2004]. While a number of possible functions of the proteosome in the regulation of Aβ metabolism have been ascribed to the multicatalytic complex of proteosomes [Guarante, 2001], additional studies are needed to understand the role of the proteosome in the clearance of Aβ. There is also a need to understand whether it is specific for the monomer, oligomer, or the protofibril, and this is not clearly known.

No reduction in the activity of γ-secretase mediated-cleavages of AβPP in the presence of resveratrol was found [Marambaud *et al*., 2005]. Thus, it excludes the possibility that resveratrol lowers Aβ by promoting the proteosomal degradation of C99 (C terminal fragment of AβPP upon cleavage by BACE). Recent evidence also suggests that Aβ can be degraded by proteosome-dependent endoplasmic reticulum (ER)-associated degradation. However, ER Aβ represents a small fraction of the total Aβ produced, and it appears to be controlled by ER-associated degradation and not resveratrol. There is no clear-cut indication on the effect of resveratrol on the mechanism of clearance of Aβ levels in the neurons, although resveratrol may have an effect on key players (components) in the Aβ clearance pathway [Marambaud *et al*., 2005]. Resveratrol was also found to interact with other proteins, including members of the sirtuin family. Sirtuins are deacetylases with a role in cellular longevity [Guarante, 2001]. It is also known that resveratrol acts as a potent activator of the human sirtuin 1 *in vitro* [Araki *et al*., 2004]. Moreover, activation of sirtuin1 by resveratrol has been linked to neuroprotective pathways [Araki *et al*., 2004]. Therefore, it would be of interest to understand whether sirtuin mediates the resveratrol-induced decrease of Aβ. However, it is not clear whether a decrease in activity of the proteosome parallels an increase of Aβ levels [Hoult *et al*., 1994]. Further, it has been shown that resveratrol selectively activates the proteosome in the anti-amyloidogenic pathway [Hoult *et al*., 1994]. Antioxidant effects of flavonoids also include transcriptional upregulation of antioxidant enzymes such as glutathione synthesizing enzymes. There is also a report interlinking the inhibitory effect of flavonoids on 5-lipoxygenase, which is involved in lipid peroxidation [Hoult *et al*., 1994]. However, it seems reasonable not to recommend alcohol intake to those who are potentially at risk for abuse and addiction [Resnick and Junlapeeya, 2004]. Fig 1.7 depicts the importance of resveratrol, in particular, in modulating neurodegeneration.

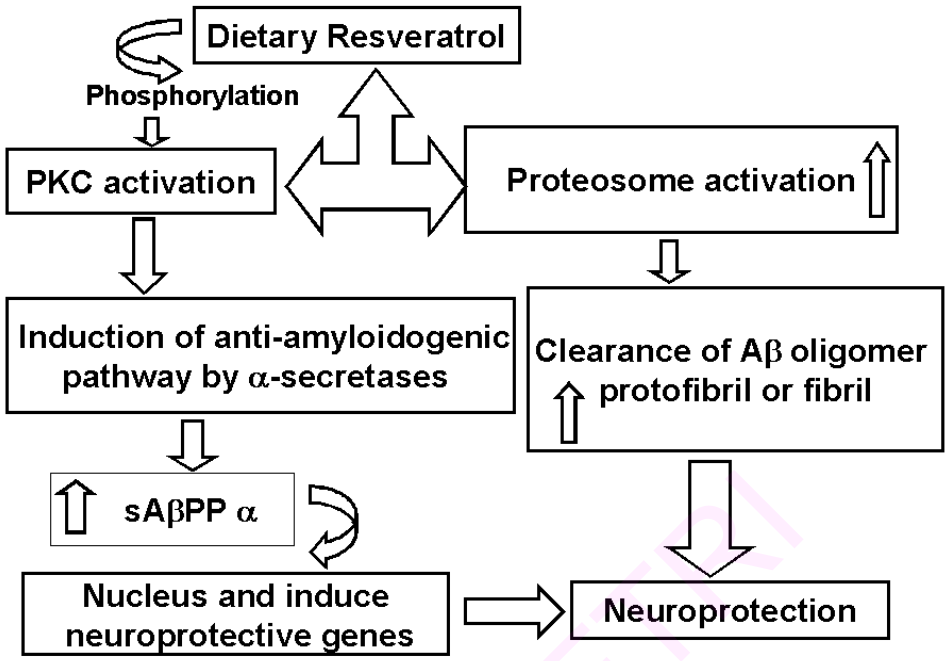


Figure 1.7: The role of resveratrol in modulating neurodegeneration. Resveratrol favors phosphorylation in PKC. This activates the non-amyloidogenic pathway of AβPP cleavage, which leads to reduction in Aβ release. sAβPPα, which is a product of AβPP cleavage, gets translocated to the nucleus and triggers the genes involved in neuroprotection. Resveratrol also nonspecifically stimulates proteosomes, which helps in clearing Aβ and reduces neuronal cell death.

Resveratrol favors phosphorylation in PKC, and this activates the non-amyloidogenic pathway of AβPP cleavage, which leads to reduction in Aβ release. sAβPPα, which is a product of AβPP cleavage, becomes translocated to the nucleus and may induce genes involved in neuroprotection. Resveratrol also nonspecifically stimulates the proteosome, which helps in clearing Aβ and in turn reduces neuronal cell death. The oral ingestion of resveratrol by rodents and humans showed that resveratrol absorbs readily into the system, appearing in plasma (total resveratrol which includes both modified and unmodified resveratrol) [Wenzel and Somoza, 2005; Gescher and Steward, 2003; Baur and Sinclair, 2006; Walle *et al*., 2006; Soleas *et al*., 2001; Vingtdeux *et al*., 2008]. The major drawback in using resveratrol in modulation of neurodegeneration is its low bioavailability [Karuppagounder *et al*., 2008]. It has been demonstrated that mice fed with feasible dosages of resveratrol for 45 days either

showed the presence of resveratrol or its metabolites in the brain indicating bioavailability to brain [Karuppagounder *et al*., 2008].

As stated above, the Mega Natural grape seed polyphenolic extract, a commercial formulation of polyphenolics derived from grape seed, significantly inhibited oligomerization of Aβ [Ono *et al*., 2008]. The extract was also shown to have the ability to inhibit the cytotoxicity of $A\beta_{40}/A\beta_{42}$ in PC12. Furthermore, the extract significantly restored the cognitive deterioration in Tg2576 transgenic mice [Ono *et al*., 2008].

1.5.8.Dietary spices and AD

The Indian diet is rich in spices including red chili, coriander, turmeric, etc. Turmeric, a yellow curry spice, is widely used as a food preservative and herbal medicine in India [Kellof *et al*., 2000], and notably, the prevalence of AD patients in India between 70 and 79 years of age is 4.4 fold less than that of the United States [Ganguli *et al*., 2000]. We hypothesize that this is partially attributed to turmeric consumption in India as a result of its curcumin contents.

Inflammation of the brain due to injury or disease is mediated by microglia [Dheen *et al*., 2007]. Brain inflammation is also mediated by activation of the complement system. AD involves a chronic central nervous system inflammatory response that is associated with both head injury and Aβ pathology [Rogers *et al*., 1996]. For example, prolonged use of non-steroidal anti-inflammatory drugs, statins and ibuprofen, have reduced inflammation in the AD brain [Breitner *et al*., 1995]. The main disadvantage of the use of non-steroidal anti-inflammatory drugs in AD is their toxicity to the gastrointestinal tract, liver, and kidney. Non-steroidal anti-inflammatory drugs are also found to inhibit cyclo-oxygenase I [Bjorkman, 1998; McGettigan and Henry, 2000]. Researchers are involved in finding alternatives to non-steroidal anti-inflammatory drugs. One such phenolic antioxidant alternative is curcumin, derived from yellow curry spice, which is found to have an anti-inflammatory effect. Curcumin is a potent free radical scavenger, better than vitamin E, and it provides protection against lipid peroxidation [MartinArgon *et al*., 1997] and acts as a scavenger of nitric oxide radicals [Sreejayan and Rao, 1997]. Curcumin also decreases the overall insoluble amyloid plaque burden in an animal model [Sreejayan and Rao, 1997]. In an intraventricular Aβ infusion rat model, dietary curcumin

reduced an isoprostane index of oxidative damage, amyloid plaque burden, and Aβ-induced spatial memory deficits in the Morris water maze [Frautschy *et al*., 2001]. Studies have shown that curcumin reduces inflammation and oxidative damage in the brain of Tg2576 AβPPSw transgenic mice [Lim *et al*., 2001; Kumar and Singh, 2008]. The low, nontoxic doses of curcumin decreases the levels of soluble and insoluble Aβ and plaque burden in many affected brain regions. Moreover, cell culture experiments with human embryonic kidney (HEK) 293 cells indicated that fibrillar Aβ (fAβ) are destabilized by nordihydroguaiaretic acid [Ono *et al*., 2002]. Thus, it may be reasonable to speculate that bioactive –molecules like curcumin, rosmanaric acid, and nordihydroguaiaretic acid could possibly prevent the onset of AD, not only by scavenging reactive oxygen species, but also by inhibiting fAβ deposition in the brain.

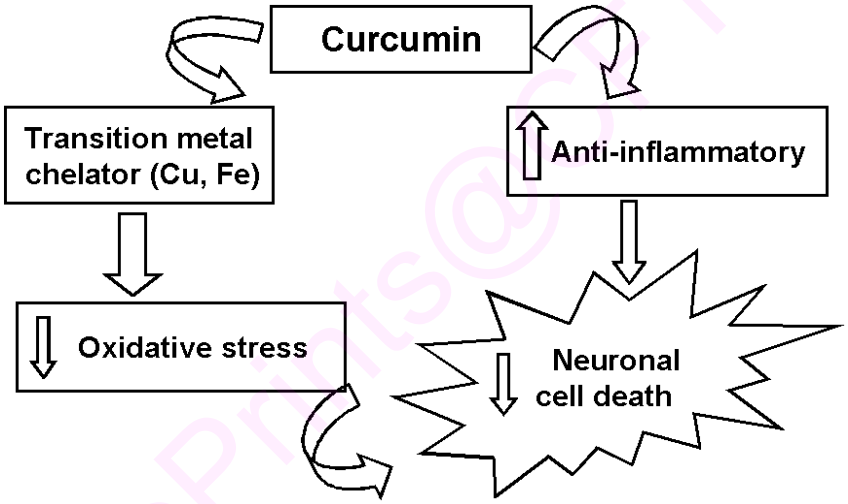


Figure 1.8: The diverse effects of curcumin in combating neurodegeneration. Curcumin has multiple biological effects. It chelates transition metals (Fe and Cu) and acts as an antioxidant and anti-inflammatory molecule, and also acts as an antioxidant by scavenging reactive oxygen species and preventing oxidative damage to macromolecules.

Curcumin also protects mouse brain from oxidative stress caused by 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine [Rajeswari, 2006] and has also been reported to attenuate 3-nitropropionic acid-induced neurotoxicity [Kumar *et al*., 2007]. In rat brain, curcumin protects against lead- and cadmium-induced lipid peroxidation as well as lead-induced tissue damage [Daniel *et al*., 2004]. Fig.1.8 highlights the diverse effects of curcumin in combating neurodegeneration. Curcumin has multiple

biological effects. It chelates transition metals (Fe and Cu) and acts as an antioxidant and anti-inflammatory molecule. It also acts as an antioxidant by scavenging reactive oxygen species, which will prevent oxidative damage to macromolecules, thereby reducing neuronal cell death.

1.5.9.Diet and genes in AD

Diet-genetic interactions may play an important role in healthy aging [Mattson, 2003]. Lifestyles include dietary patterns during early, middle, and adult life that may influence the risk of brain disorders. Genetic factors include mutations in genes like amyloid precursor protein, presenilin 1, and presenilin 2 that are risk factors and cause early onset events in AD. Research related to the effect of dietary molecules on the expression of AβPP gene and its processing to produce Aβ has not been done. The scope of the dietary molecules on AD can also be extended for stimulating Aβ clearance mechanisms of cells. APOEε4 is the most important genetic risk factor for AD and cardiovascular diseases [Corder *et al*., 1993; Hofman *et al*., 1997]. Dietary molecules may affect genes independently or through signaling molecules. APOE ε4 also been associated with cognitive decline [Hara *et al*., 1998; Caselli *et al*., 1999]. The genotype APOE ε4 has a differential effect on fat consumption in different stages of human life. Importantly, there are limits to commenting on dietary-gene interactions and more data is required to arrive at conclusion.

1.6.Drug discovery in AD

The drug discovery programs in AD targeted the beta-amyloid mainly and others include metals, inflammatory molecules, oxidative stress and etc. The ultimate goal of therapeutic intervention is either to halt or to slow down the AD progression. The beta-amyloid plays a central role in onset and also progression of pathophysiology of AD. The therapeutics of AD rolls around beta-amyloid. In depth research has been focused in studying the pathophysiology of beta-amyloid in cell culture system, animal models and in humans. The four ways of targeting beta-amyloid include; (i) decrease amyloid production, (ii) increased degradation (iii) increased clearance (iv) inhibiting aggregation and / toxicity to neurons in central nervous system. The inhibitors of beta-secretase and gamma-secretase have been associated with side effects. So, this raised concerns about their therapeutic potential [Chow *et al*., 2010]. Chow *et al*. [2010] demonstrated the genetic reduction of both BACE1 and gamma

secretase additively abolished the amyloid load burden and decrease cognitive decline in aged APPSwe/ PS1 E9 animals. The decrease in both enzymes are not found to be associated with mechanistic toxicities [Chow *et al*., 2010]. The presently available drugs for AD include acetylcholine esterase inhibitors and N-methyly-D-aspartate receptor agonist and these will not halt the pathophysiology of AD [Scarpini *et al*., 2003]. Colostrinin is polypeptide rich in proline obtained from sheep colostrum. The colostrinin is found to inhibit the beta-amyloid aggregation *in vitro* [Gibson *et al*., 2004; Schuster *et al*., 2005]. The clioquinol is a copper metal chelator which has shown promise in reducing amyloid aggregation and there by amyloid and consequent increase in the cognitive performance. But this drug has been withdrawn from the market because of its myelo-optic- neuropathy [Ritchie *et al*., 2003]. NSAIDS: The epidiomologic studies suggest that long-term use of NSAIDs benefits AD patients [int Veld *et al*., 2001; McGeer *et al*., 1996; Szekely *et al*., 2004]. Some of the NSAIDs such as rofecoxib, naproxen and diclofenae did not stop or slow down the progression of mild AD to AD [Aisen *et al*., 2003; Reines *et al*., 2004; Scharf *et al*., 1999]. Additionally, indomethacin though delayed cognitive decline but it has shown to have gastro-intestinal toxicity, myocardial infaraction and stroke [Rogers *et al*., 1993; Tabet and Feldman, 2002]. So, NSAIDs are not considered for treating AD patients. Statins: The intake of statins for long duration is known to improve the conditions in AD patients [Wolozin *et al*., 2000]. The mechanism of statins induced protection of AD is not understood clearly but it is suggested that it reduces serum cholesterol and also has anti-inflammatory properties [Sparks *et al*., 2005]. The other activities of statins include inhibition of HMG-CoA reducates activity. The HMG-CoA reductase activity inhibition is known to increase the activity of α- secretase, which results in abolishing the beta-amyloid release from APP. So, statins believed to act through Rho associated protein kinase 1(Rock1). The Rock1 modulates α- secretase activity [Pedrini *et al*., 2005]. Recently there are two drugs that are undergone clinical trials both are targeted to beta-amyloid metabolism. The bapineuzumab is an antibody to beta-amyloid and it was used for passive immunization in phase 2 trials. The mode of action of bapineuzumab is that it clears aggregated beta-amyloid by activating the astrocytes mediated phagocytosis. The bapineuzumab is an anti-beta amyloid monoclonal antibody. It has been known to bind to beta-amyloid plaques and reduces

amyloid burden in mouse model of AD [Bard *et al*., 2000]. Additionally, reduction in amyloid correlated to reversal of memory deficits [Dodart *et al*., 2002]. The pahse 2 trials involving bapineuzumab treated for 78 weeks reduced the fibrillar amyloid burden in human AD subjects as shown by PiB-PET [Rinne *et al*., 2010]. The tarenflurbil is employed for lowering beta-amyloid by decreasing gamma-secretase and consequently improves memory and learning in mouse model of AD [Kukar *et al*., 2007]. In phase 2 trial involving 210 AD patients were administered 800mg tarenflurbil twice a day had increased routine activities and global function compared to placebo-received subjects [Wilcock *et al*., 2008]. The positive outcomes of phase 2 trials of tarenflurbil, it is forwarded for phase 3 trials. The out come of the phase 3 trial involving tarenflurbil failed to improve primary and secondary symptoms. Additionally, the subjects who have received tarenfrurbil have showed symptoms of dizziness, anemia, respiratory infection, increase in blood pressure and development of rashes. So, the phase 3 trials have been stopped. The results of clinical trails of bapineuzumab and tarenflurbil has following implications; though there is a reduction in beta-amyloid load or aggregated amyloid, other forms of beta-amyloid such as oligomeric forms may still cause neurotoxicity; (ii) the reduction in amyloid may not be effective in patients whose AD pathophysiology is no more dependent on amyloid load. The drawback of amyloid hypothesis is that it did not consider for the cerebrovascular damage in the cause of neurodegeneration [De Leeuw *et al*., 2006; Prins *et al*., 2010]. The disadvantage of the use of synthetic drugs being shown to have many side effects. There comes the importance of natural products having no or less side effects. The natural products have shown to be potential for preventing many neurodegenerative diseases.

1.7. Aim and scope of the study

Keeping the above literature in view, he present study is aimed, i) to understand the effects of different fragments induced genotoxicity, ii) *in vivo* effects of beta-amyloid (1-42) DNA stability and conformation are studied using aged rabbits, iii) indigenous plants such as *C. crista* and *C. asiatica* aqueous extract to prevent beta-amyloid aggregation. These studies provide novel mechanisms of amyloid beta peptide in AD neurodegeneration.

Objectives:

1.To study the mapping of amyloids induced genotoxicity.

2. *In vivo* studies using aged rabbits to understand the neuroprotective role Indigenous plants in amyloids induced genotoxicity in relevance to brain tomography and DNA damage.

3. To study anti-amyloidogenic properties of indigenous medicinal herb.

Chapter 2

Studies to map amyloidogenic peptides induced genotoxicity

Chapter 2 Studies to map amyloidogenic peptides induced genotoxicity

2.1. Introduction

Alzheimer's disease (AD) is a common form of dementia and is characterized by loss of memory, inability to perform daily activities, language impairment and behavioral abnormalities [Bose *et al*., 2005; Citron, 2010]. AD is characterized by the extracellular accumulation of Aβ as senile plaques (SP) and intracellular neuroibrillary tangles (NFTs) [Selkoe, 2001]. The Aβ is attributed to cause the neuronal death by more *in vitro* [Liu *et al*., 2009; Kaneko *et al.,* 1995; 2001; Zeng *et al*., 2004] but the mechanisms are not still clear. Aβ in suitable medium, will self–assembles to form oligomers, protofibrils and matured fibrils [Chomy *et al*., 2003]. Many of these forms are neurotoxic in cell culture models [Cizas *et al*., 2010; Gustavsson *et al.,* 1991; Castano *et al* 1986; Guillozet *et al.* 2003]. Currently, the widely accepted hypothesis is that the oligomeric form of Aβ are more toxic than the insoluble fibrillar form [Cizas *et al*., 2010; Lambert *et al*., 1998; Klein, 2001; Urbanc *et al*., 2010; Kelly and Ferreira, 2007]. The oligomeric form of Aβ is reported to inhibit the hippocampal long-term potentiation (LTP) *in vivo* [Walsh *et al*., 2002]. The mechanisms of neurotoxicity induced by Aβ are controversial and there is no unified hypothesis. But limited studies have shown that Aβ activate nuclear factor κB (NF-κB) and induces apoptosis [Akama, 1998; Ghribi *et al.,* 2001]. There are studies that indicated the nuclear localization of Aβ and DNA binding abilities of Aβ **[**Gouras *et al*., 2000; Suram *et al* ., 2007; Hegde *et al*., 2004]. These new studies indicate that Aβ induces neurotoxicity though nicking DNA, changes in DNA conformation and DNA stability [Zeng *et al*., 2004; Pillot *et al* ., 1999; Blanc *et al*., 1997; Yu *et al.,* 2007; Suram *et al* ., 2007; Hegde *et al*., 2004]. The studies from our lab and others, have demonstrated nuclear localization of Aβ(1-42) in AD brain [Hegde *et al.,* 2004; Gouras *et al*., 2000]. Ohyogi *et al*. [2005] showed Aβ(1-42) localization to cytoplasm and nucleus in primary neurons of guinea pigs transfected with constructs of Aβ42/Aβ40. Further, Bucking *et al*. [2002] showed that Aβ(1-42) is overproduced in ER and translocated into cytosol and partly into to nucleus, but the mechanism is still not clear [Johnstone *et al*., 1996]. The Aβ(1-42) found in nucleus known to activate

p^{53} dependent apoptosis [Ohyogi *et al*., 2005]. Ohyogi *et al*. [2005] reported that Aβ(1-42) in the nucleus will act as transcription like factors in association with death inducing factor. Theoretically, Aβ forms β-hairpin shape followed by a helix–turn-helix motif [Durrel *et al*., 1994] that may be an essential motif in Aβ to form DNA binding domain and this kind observation is seen in heat shock transcription factor. And p^{53} promoter region contains heat shock elements [Wu, 1995] and Aβ42 might directly bind p^{53} promoter region and causes DNA damage [Sun *et al*., 1995]. The oligonucleotides derived from p^{53} promoter is interacted with Aβ(1-42) and showed that Aβ(1-42) binds to oligonucleotides of p^{53} promoter [Ohyogi *et al.,* 2005]. Earlier reports by Hegde *et al*. [2004] showed that Aβ(1-42) relaxed Supercoiled (Sc) DNA completely, while Aβ(1-16) partially relaxed of ScDNA. Additionally, Aβ(1-42) could also induce the conformation of B-form of ScDNA to ψ-DNA, which closely resembles Z-DNA [Hegde *et al*., 2004). The studies using fluorescence microscopy showed that cells treated with Aβ have condensed DNA [Zeng *et al*., 2004; Blanc *et al*., 1997]. Suram *et al*. [2007] for the first time showed that Aβ(1-42) causes instability of ScDNA by nicking [Suram *et al*., 2007]. Yu *et al*. [2007] reported that Aβ(40) induces time dependent DNA condensation and further Cu and Zinc inhibits the DNA condensation. These studies indicate that the exposure of Aβ to neuronal cells cause DNA strand breaks, this may be one of the mechanisms of Aβ induced genotoxicity [Santiard-Baron *et al*., 1999]. But the mechanisms of DNA nicking by Aβ is still not clear. In the present study, we propose to map the DNA nicking ability of different fragments of Aβ. In the present study, we have used both N-terminal and C-terminal fragments of Aβ [Aβ(1-11,1-28, 22-35, 25-35, 17-42, 1-40, 1-42 and 1-43] to under stand the mechanism of Aβ nicking DNA.

2.2. Materials and methods

Supercoiled plasmid DNA (pUC18) (Cesium chloride purified) and DNA molecular markers were purchased from Bangalore Genei, India. Aurin tricarboxylic acid (ATA) and Aβ(1-11), Aβ(1-28), Aβ(22-35), Aβ(25-35), Aβ(17-42), Aβ(1-42) and

Aβ(1-43) were purchased from Sigma, USA. TRIS (hydroxymethyl) and agarose from SRL India. Glacial acetic acid and $MgCl_2$ were purchased from Himedia chemicals, India. Ethidium bromide was obtained from ICN.

2.2.1. ScDNA nicking activity of different fragments of Aβ

ScDNA (0.5µg) was incubated with 50µM of different fragments of Aβ [Aβ(1-11,1-28, 17-42, 22-35, 25-35, 1-40, 1-42, 1-43)] for 12 h in Tris-Cl buffer (pH 7.4) at 37^0C. Incubated samples were electrophoresesed on 1% agarose using TAE (Tris – acetic acid -EDTA) (pH8.4). The gel was stained with 1µg/ml of ethidium bromide in cold water for one h and de-stained using triple distilled water and documented using gel documentor and densitometry was recorded using the inbuilt software system.

2.2.2. Modification of histidine at 11th position in Aβ (1-11) by DEPC and its effects on different forms of DNA(ScDNA, λDNA, Single stranded circular DNA and double stranded circular DNA)

Histidine in the 11th position of Aβ (1-11) was modified using diethyl pyrocarbonate (DEPC) method (modified protocol from Atwood *et al*., 1998). 100µM of Aβ (1-11) was incubated with 10mM of DEPC in 100mM of KH_2PO4 (pH 6.5) for 30 min at room temperature. The modified histidine, N-carbethoxyhistidine gives absorbance maxima at 240nm. The DEPC remaining in the reaction mixture was removed by using the G-10 sepharose column. Since the DEPC has low molecular weight and will be eluted at the end, where as modified Aβ (1-11) eluted before DEPC. The fraction having highest OD at 240nm were pooled and lyophilized. The lyophilized powder was dissolved in 10mMTris-Cl, (pH7.4) and used for DNA nicking study. 0.5µg of ScDNA, λDNA, Single stranded DNA and double stranded DNA were incubated with Aβ(1-11) and M Aβ(1-11) for 12 h in Tris-Cl buffer (pH7.4) at 37^0C. Incubated samples were electrophoressed on 1% agarose using TAE (Tris –acetic acid -EDTA) (pH 8.4). The gel was stained with 1µg/ml of ethidium bromide in cold water for one h and de-stained in using triple distilled water and documented using gel documentor.

2.2.3. Effect of Mg^{2+} on DNA nicking activity of different fragments of Aβ

ScDNA (0.5µg) was incubated with 50µM of different fragments of Aβ [Aβ(1-11,1-28 , , 17-42, 22-35, 25-35, 1-40, 1-42, 1-43)] in presence of 1mM $MgCl_2$ for 12 h in Tris-Cl buffer at pH7.4 at 37^0C. Incubated samples were electrophoressed on 1% agarose using TAE (Tris–acetic acid-EDTA) (pH8.4). The gel was stained with 1µg/ml of ethidium bromide in cold water for one h and destained in using triple distilled water and documented using gel documentor and densitometry was recorded using the inbuilt software system.

2.2.4. Effect of ATA on DNA nicking activity of different fragments of Aβ

ScDNA (0.5µg) was incubated with 50µM of different fragments of Aβ [Aβ(1-11,1-28 , , 17-42, 22-35, 25-35, 1-40, 1-42, 1-43)] in presence of both 500µM ATA and 1mM $MgCl_2$ for 12 h in Tris-Cl buffer (pH7.4) at 37^0C. Incubated samples were electrophoressed on 1% agarose using TAE (Tris –acetic acid -EDTA) (pH8.4). The gel was stained with 1µg/ml of ethidium bromide in cold water for one h and destained in using triple distilled water and documented using gel documentor and densitometry was recorded using the inbuilt software system.

2.2.5. Ethidium bromide binding studies

To know the extent of DNA damage by the Aβ fragments, we have quantified the ethidium bromide (EtBr) binding to ScDNA. The quantification of number of Etbr bound per base pair of ScDNA was measured in 0.01mM Tris-Cl, pH7.4 using HITACH F-2000 fluorescence spectrophotometer. The fluorescence was measured using constant amount of DNA (0.5µg) with 50µM of different Aβ fragments incubated for 12 h at 37^0C. The incubated sample was titrated with increasing amount of EtBr against the blank containing no DNA. The measurements were made keeping the excitation at 535 nm and emission at 600nm with 10mm path length. The maximum EtBr bound per base pair was calculated using the Scatchard plots of 'r' vs. 'r/Cf' in the DNA-EtBr reaction mixture at various intervals when increasing amounts of EtBr was titrated to constant amount of ScDNA [Scatchard, 1949]. The concentration of bound EtBr one mL dye-DNA mixture ('Cb') was calculated using the equation:

Cb'=[C_o (F-FO)/(V/Fo)], where

Co =concentration of EtBr (pmoles) in the dye complex mixture

F= observed fluorescence of EtBr at any point of dye-DNA mixture

Fo= Observed fluorescence of EtBr with no DNA

V=Experimental value, ratio of bound EtBr to free EtBr at saturation point.

The concentration of free dye (Cf') was then calculated by using the formula

$$Cf' = Co' - Cb'$$

Where, Cf, Co'and Cb' were expressed in pmoles. The amount of bound EtBr bound per base pair was calculated by

r=Cb' (pmoles)/DNA concentration (pmoles of base pair).

2.2.6. Melting temperature(Tm) studies

To record the change in the stability of the ScDNA induced by different Aβ fragments using melting profiles of ScDNA. The ScDNA (5µg) was incubated with 50µM of different Aβ fragments in 0.01mM of HEPES, pH 7.4 for 12 h at 37^0 C. The incubated samples were used to record the melting profiles of ScDNA using the spectrophotometer equipped with thermoprogramer and data processor (Amersham, Hong Kong). The hyperchomicity of ScDNA was recorded from 45^0C- 95 ^{0}C with 1^0C per minute. The temperature point at which there was a 50% hyperchromic shift was taken as Tm of the ScDNA sample. Tm values were determined graphically from the hyperchromicity vs. temperature plots.

2.2.7.Circular dichoism spectroscopy to study the effect of histidine modified Aβ (1-11) on conformation of ScDNA

15µg ScDNA was incubated with 50µM of modified Aβ (1-11) and without modified Aβ (1-11) for 12 h in 10mM Tris-Cl, (pH7.4.)The conformation of ScDNA was recorded on JASCO J 700 Spectropolarimeter at 25 ^{O}C, with 2mm cell length with a wave length scan between 200nm and 320nm.

2.2.8. Circular dichoism spectroscopic study of different fragments of Aβ(1-11, 1-28, 25-35 and 1-43)

50μM of different fragments of Aβ[1-11, 1-28, 25-35 and 1-43] incubated with for 12 h at 37 ^{O}C in 10mM Tris-Cl, pH7.4. The secondary conformation of different fragments of Aβ was recorded on JASCO J 700 Spectropolarimeter at 25 ^{O}C, with 2mm cell length with wavelength scan between 195 and 260nm.

2.2.9. Protscale analysis of the Aβ(1-43, 1-42, 1-11 and 25-35)

Protscale was developed by Prof. Joel Susman of Weizmann Institute of Israel. The protscale was a free online program. It would provide information on the contribution of amino acid residues to the conformation of proteins such as α- helix, β- sheet and random coil. The selected sequence of protein need to be deposited on the particular window provided and select the particular conformation and click on submit. The window size for Aβ (1-11 and 25-35) was 5 where as for Aβ(1-42 and 1-43) it was 9. The relative weight of window edges compared to the window center (in %) is 100%. The weight variation model (if the relative weight at the edges is (< 100%) is linear. The the scale was normalized from 0 to 1.The computer data base output will provide the information on the contribution of different amino acid residues to particular conformation of the protein in question in the form graph and values. Here we represented the only graph. We have the analysed the amino acid sequences of Aβ[(1-11, 25-35, 1-42 and 1-43)] because of their biological importance.

2.3. Results

2.3.1. Evidence for linearization of ScDNA by different fragments of Aβ

The agarose gel study was done to demonstrate the formation of linear and open circular forms of DNA from ScDNA as a result of nicking activity of different fragments of Aβ. Fig.2.3.1a shows the DNA nicking property of different Aβ fragments. Lane 1 represents the 1kb marker (molecular weight markers from top to bottom are as follows 10,000,8000,7000,6000,5000,4000,3000,2000,1000) and lane 2 represents the linearized ScDNA (EcoR1 digested). Lane a represents the ScDNA alone with 85% supercoiled DNA as shown by the intense supercoiled DNA band (form I) and 15 % open circular form. Aβ(1-11), Aβ(1-28), Aβ(1-40) and Aβ(1-42)

nicked ScDNA and converted to ScDNA to both open and linear form. But Aβ(22-35), Aβ(25-35), Aβ(17-42) and Aβ(1-43) did not nick ScDNA. The densitometry values of the agarose gel (fig 2.3.1a) was plotted using the bar diagram (fig 2.3.1b).

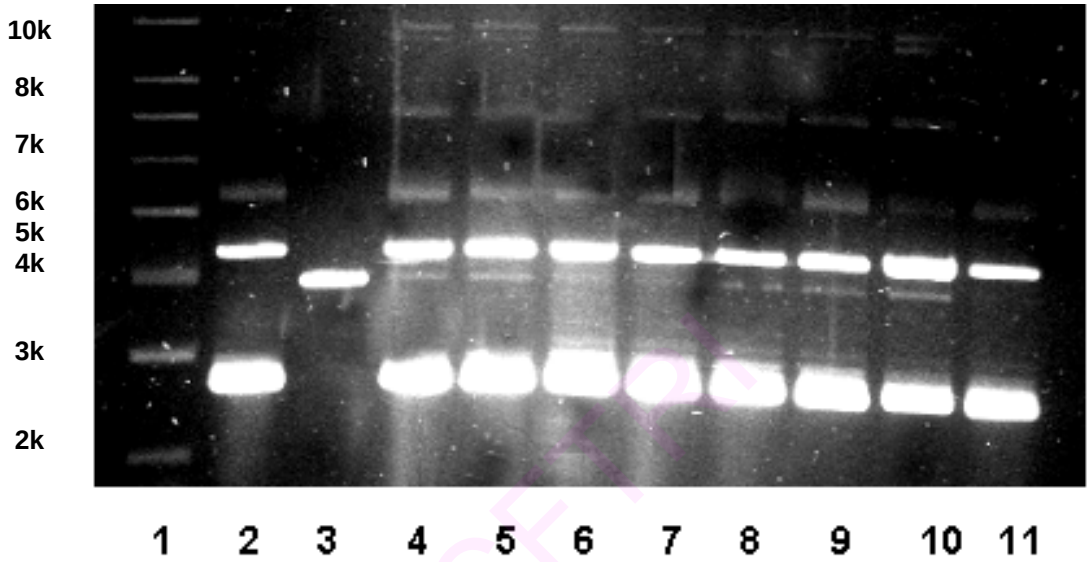


Fig 2.3.1a: Agarose gel electrophoresis showing DNA nicking property of different Aβ fragments: 1-1kb marker, 2- Linearized DNA-EcoRI digested pUC 18 DNA, 3- ScDNA, 4-ScDNA+ Aβ (1-11), 5- ScDNA+ Aβ (1-28), 6- ScDNA+ Aβ (22-35),7- ScDNA+ Aβ (25-35), 8- ScDNA+ Aβ (17-42), 9- ScDNA+ Aβ (1-40), 10- ScDNA+ Aβ (1-42), 11- ScDNA+ Aβ (1-43).

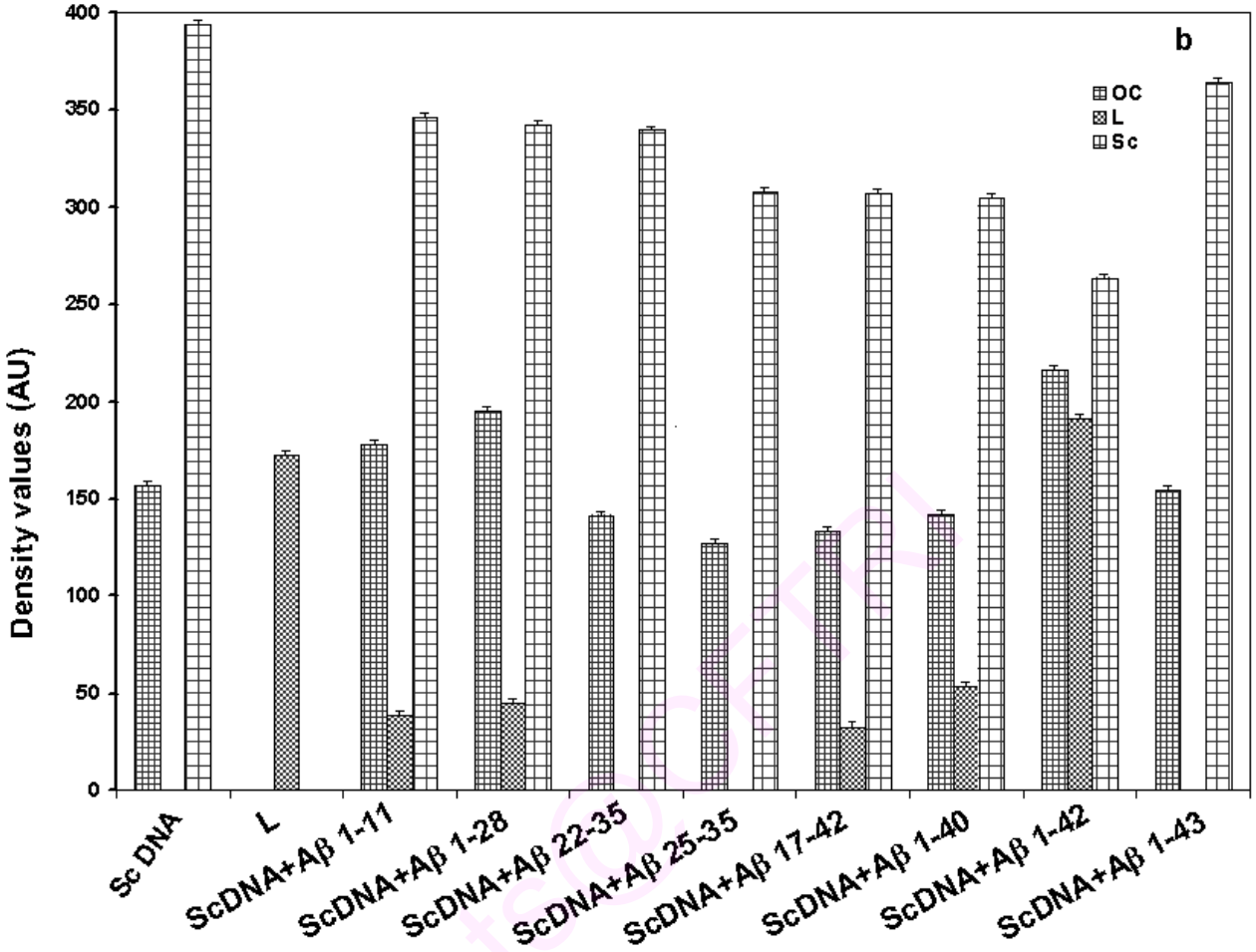


Fig 2.3.1b: Bar diagram showing densitometry values of agarose gel (Fig 2.3.1a)

2.3.2. Effect of Aβ(1-11) and M Aβ(1-11) on the ScDNA, λDNA, Single stranded circular DNA and double stranded circular DNA

We hypothesized histidine involvement in the DNA nicking property of Aβ fragments. To prove this hypothesis we have selected Aβ(1-11) and modified the only histidine present at 11th position using DEPC method. Aβ(1-11) has nicked the ScDNA while the histidine modified Aβ(1-11) did not nick the ScDNA but it has shown retardation of ScDNA. Aβ(1-11) and M Aβ(1-11) has retarded the λDNA. But both unmodified and modified Aβ(1-11) peptide did not nick λDNA. Aβ(1-11) Did

not nick single stranded and double stranded circular DNA and did not retard, where as histidine modified Aβ(1-11) did not nick single stranded and double stranded circular DNA but retard the single stranded and double stranded circular DNA(fig 2.3.2).

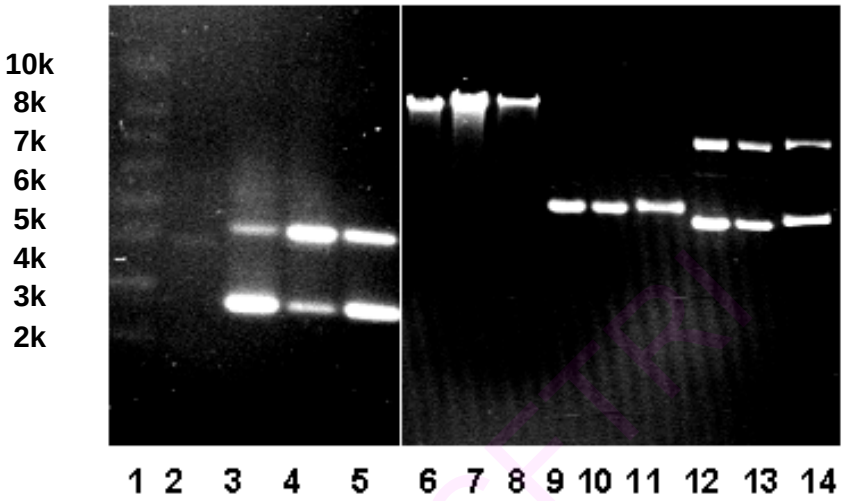


Fig 2.3.2: Agarose gel showing the histidine modified Aβ(1-11) on different forms of DNA: 1- 1kb marker, 2- Linearized DNA-EcoRI digested pUC 18 DNA, 3-ScDNA, 4- ScDNA+ Aβ(1-11), 5- ScDNA+ Aβ(1-11)M, 6- λDNA,7- λDNA + Aβ(1-11), 8-λDNA + Aβ(1-11)M, 9- SSC DNA, 10- SSC DNA+ Aβ(1-11), 11- SSC + Aβ(1-11)M, 12- DSC DNA, 13-DSCDNA + Aβ(1-11), 14- DNA + Aβ(1-11)M.

2.3.3.Effect of Mg^{2+} on DNA nicking activity of different fragments of Aβ (1-42)

The metal ions Mg act as cofactors for endonucleases. The Mg^{2+}enhanced the DNA nicking activity of Aβ(1-42) [Suram *et al*., 2007]. Mg^{2+} has enhanced the DNA nicking activity of Aβ(1-11, 1-28, 1-40 and 1-42). In presence of Mg^{2+} Aβ(22-35, 25-35, 17-42 and 1-43) nicked ScDNA and converted ScDNA to open circular and linear forms. (Fig 2.3.3a). So Mg^{2+} has differential effect on endonuclease activity of Aβ fragments. The densitometry values of the agarose gel was plotted using bar diagram (fig 2.3.3b)

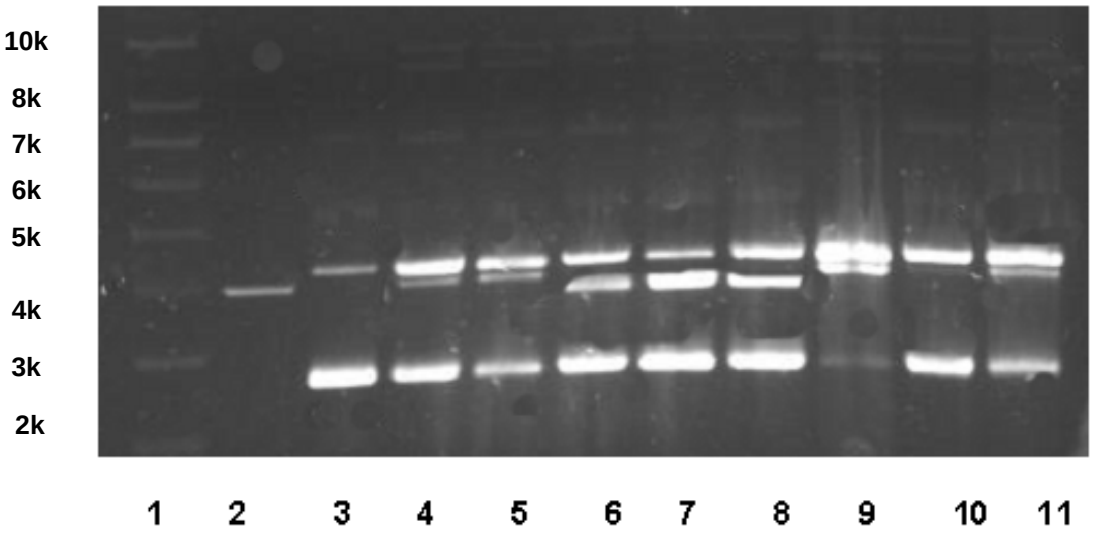


Fig 2.3.3a: Agarose gel showing the effect of Mg^{2+} on DNA nicking activity of different fragments of Aβ: 1-1kb marker, 2- Linearized DNA-EcoRI digested pUC 18 DNA, 3- ScDNA, 4-ScDNA+ Aβ (1-11)+ Mg^{2+}, 5- ScDNA+ Aβ (1-28) + Mg^{2+}, 6- ScDNA+ Aβ (22-35)+ Mg^{2+}, 7- ScDNA+ Aβ (25-35)+ Mg^{2+}, 8- ScDNA+ Aβ (17-42)+ Mg^{2+}, 9- ScDNA+ Aβ (1-40)+ Mg^{2+}, 10- ScDNA+ Aβ (1-42)+ Mg^{2+}, 11- ScDNA+ Aβ (1-43)+ Mg^{2+}.

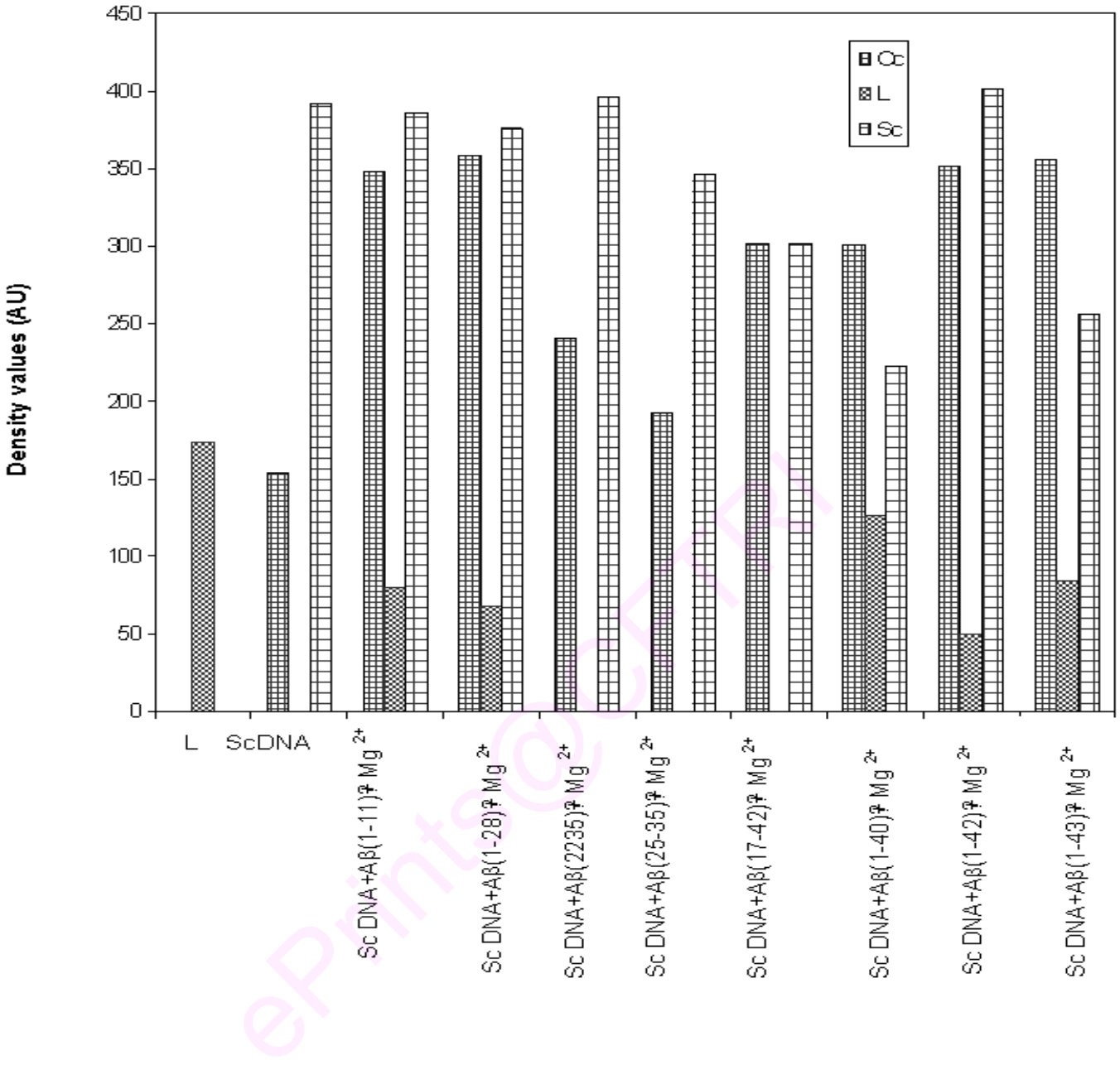


Fig 3.3.3b : **Bar diagram showing densitometry values of agarose gel (Fig 3.3.3a)**

2.3.4. Inhibition of Aβ ScDNA nicking activity of ATA

ATA is a specific nuclease inhibitor [Hallick *et al* 1977]. ATA only inhibited the Sc DNA nicking activity of Aβ(1-42), where as ATA in presence of Mg^{2+} enhanced nicking activity of Aβ(1-11, 1-28, 22-35, 25-35, 17-42, 1-40 and 1-43) (Fig.2.3.4a). The densitometry values of agarose gel (fig 2.3.4a) was represented using the bar diagram (Fig 2.3.4b).

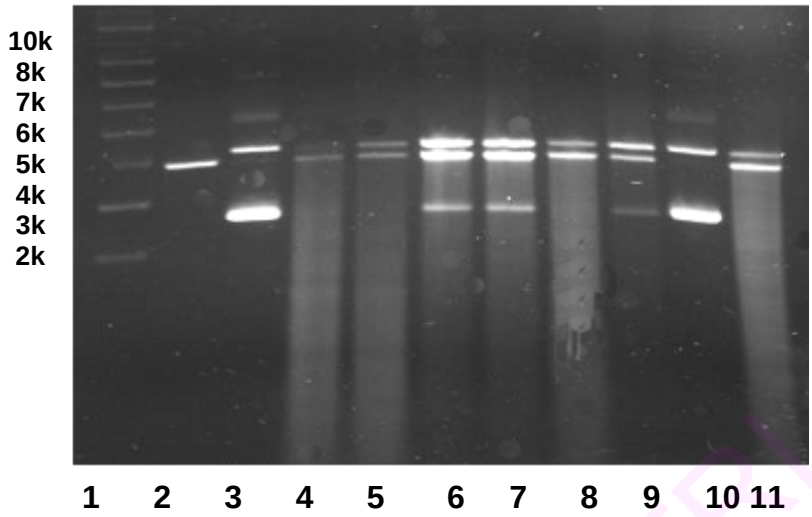


Fig 3.3.4a: Agarose gel showing effect of ATA on DNA nicking property of different fragments of Aβ: 1-1kb marker, 2- Linearized DNA-EcoRI digested pUC 18 DNA, 3- ScDNA, 4-ScDNA+ Aβ (1-11)+ Mg^{2+}+ ATA, 5- ScDNA+ Aβ (1-28)+ Mg^{2+}+ ATA, 6- ScDNA+ Aβ (22-35)+ Mg^{2+}+ ATA, 7- ScDNA+ Aβ (25-35)+ Mg^{2+}+ ATA, 8- ScDNA+ Aβ (17-42)+ Mg^{2+}+ ATA, 9- ScDNA+ Aβ (1-40)+ Mg^{2+}+ ATA, 10- ScDNA+ Aβ (1-42)+ Mg^{2+}+ ATA, 11- ScDNA+ Aβ (1-43)+ Mg^{2+}+ ATA.

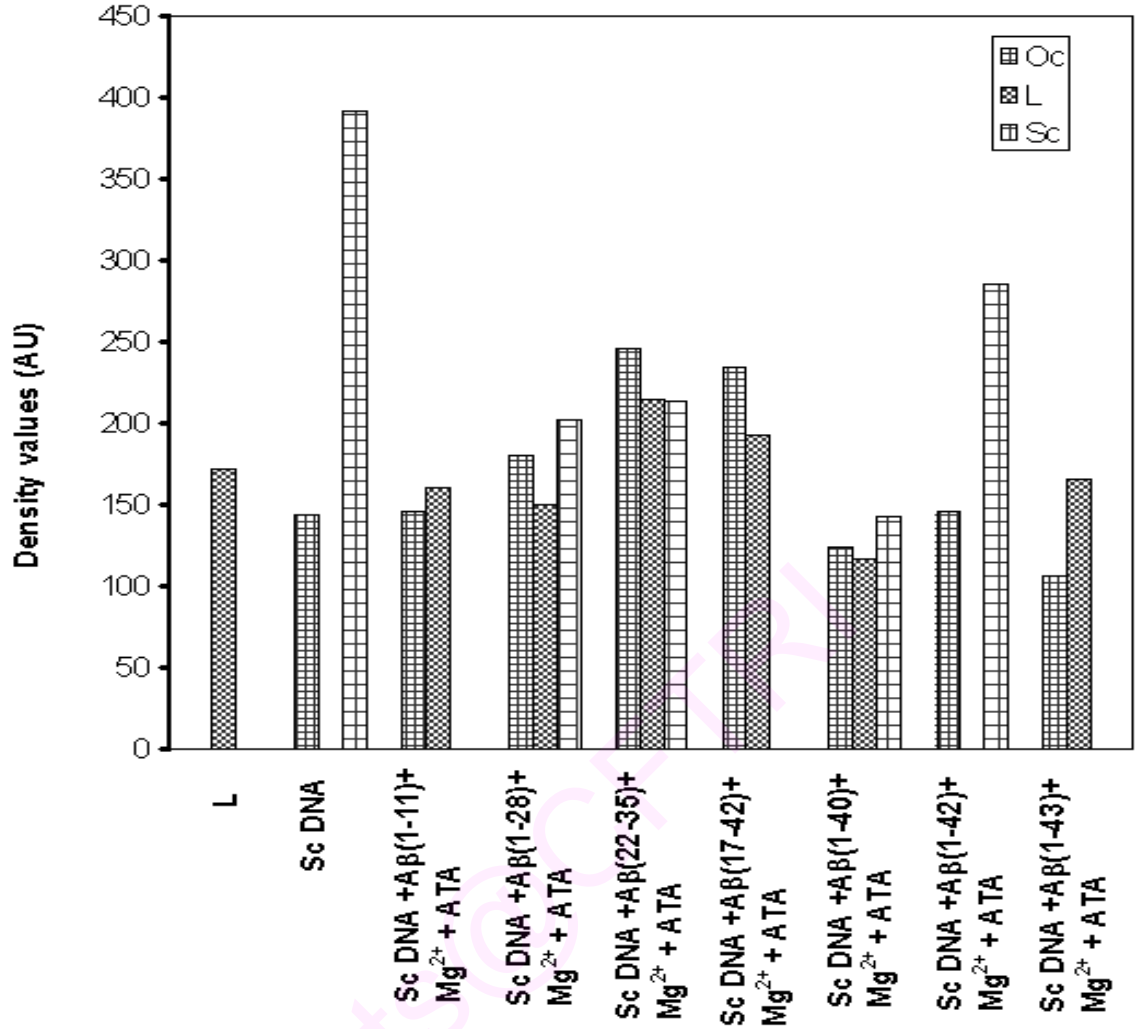
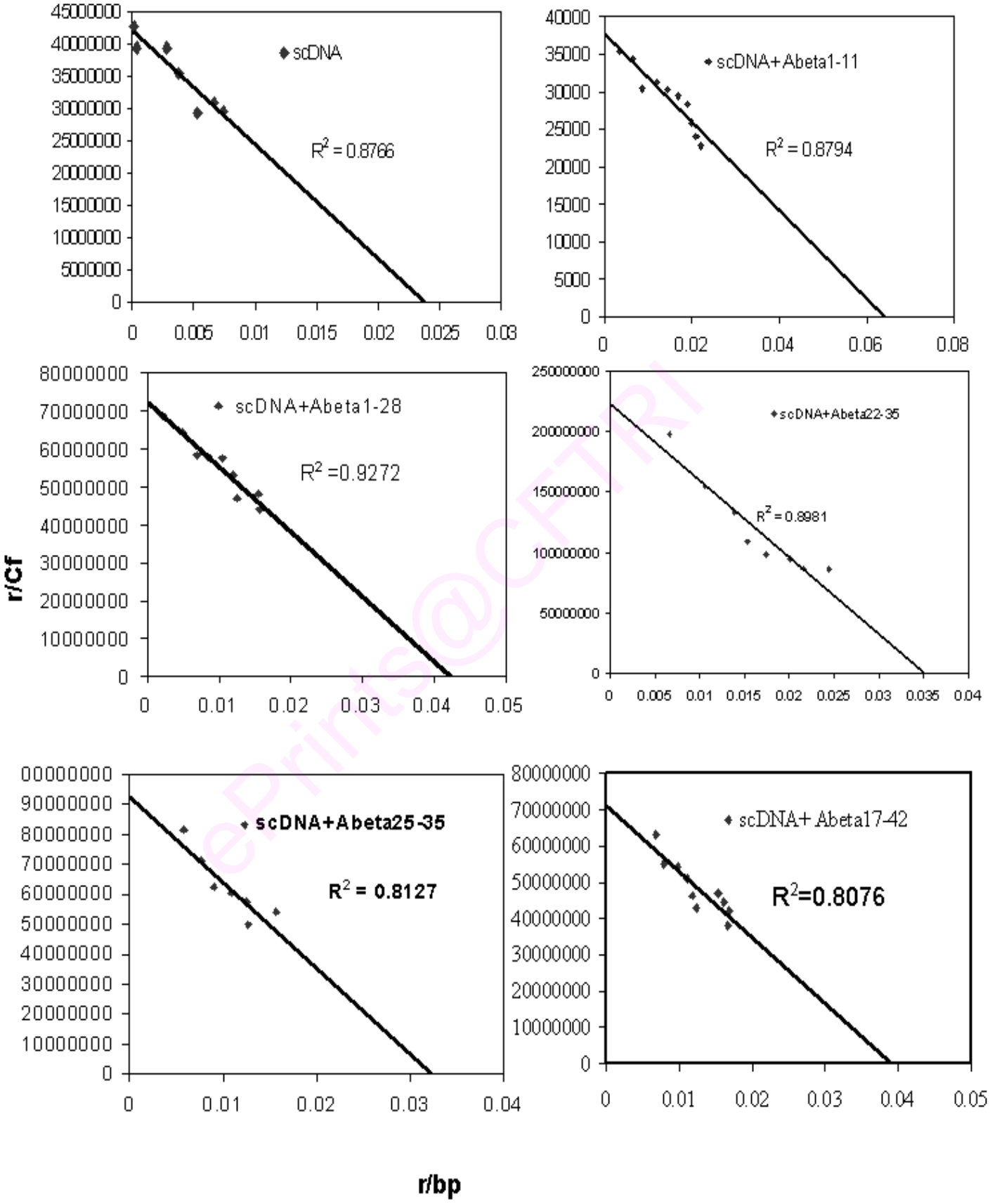


Fig 2.3.4b: Bar diagram showing densitometry values of agarose gel(Fig 2.3.4a)

2.3.5. Effect of different fragments of Aβ on ethidium bromide binding to ScDNA

The EtBr binding studies indicate DNA stability and Scatchard plot analysis shows the number of EtBr bound per base pair (bp) of DNA(fig 2.3.5). The experiment was aimed to understand the effect of Aβ on number of ethidium bromide (Etbr) binding to ScDNA shown in table 2.1.The increasing in the number of Etbr binding to ScDNA was observed when it was interacted with Aβ(1-11), Aβ(1-28), Aβ(1-11), Aβ(22-35), Aβ(25-35), Aβ(17-42), Aβ(1-42), and decreased EtBr binding to ScDNA when it is interacted with Aβ(1-40), Aβ(1-43).



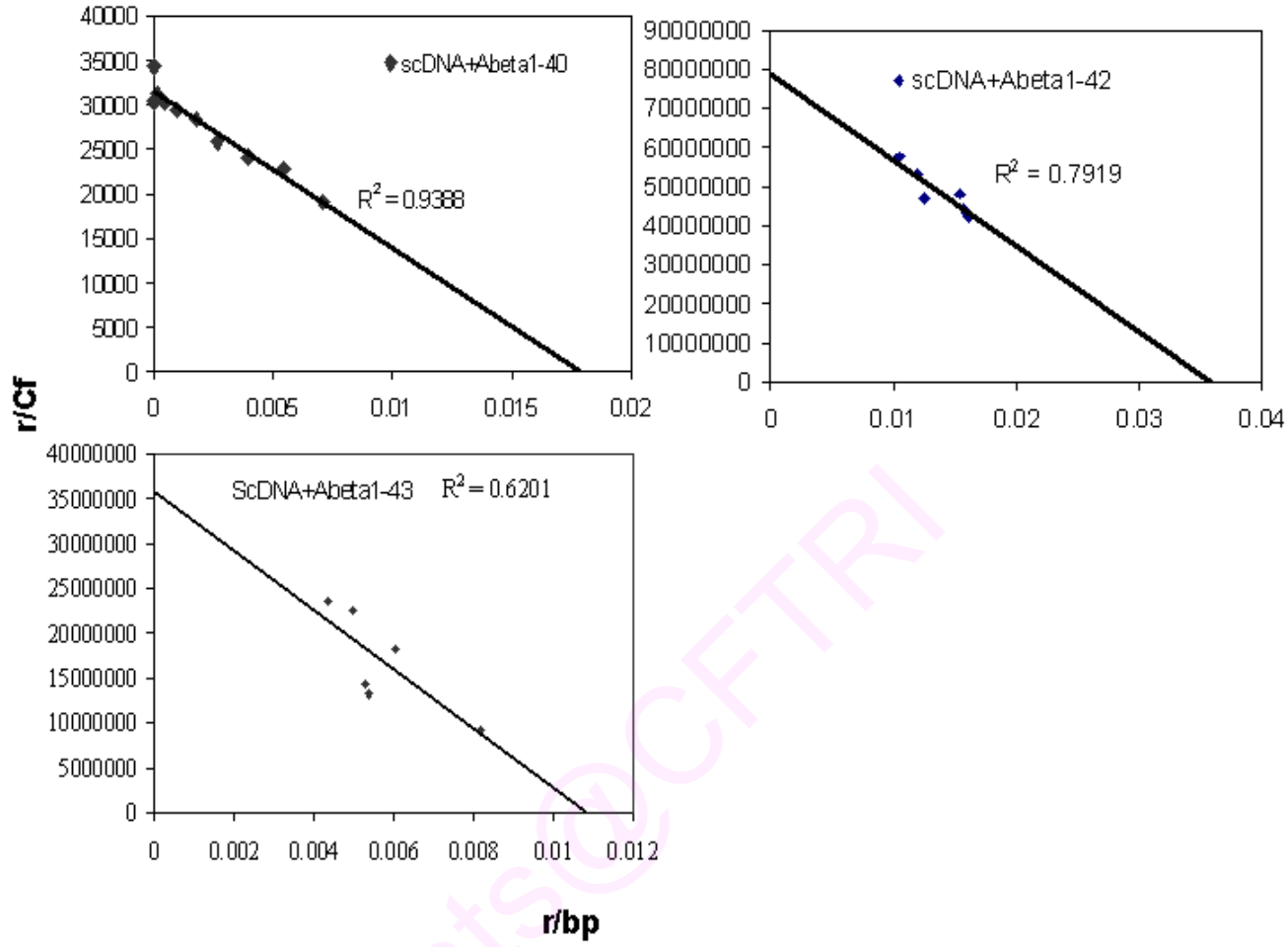


Fig 2.3.5: Scatchard plot showing number of EtBr molecules per base pair of ScDNA. ScDNA was interacted for 12 h with different fragments of Aβ and EtBr binding was quantified using Scatchard plot.

Table: 2.1: The data indicates the number of EtBr molecules binding per base pair of DNA interacted with different fragments of Aβ.

Sl No	ScDNA+ Aβ fragments	No.EtBr /Bp
1	Sc DNA alone	0.024
2	Sc DNA+Aβ(1-11)	0.062
3	ScDNA+ Aβ(1-28)	0.042
4	Sc DNA+ Aβ(22-35)	0.035
5	Sc DNA+ Aβ(25-35)	0.032
6	Sc DNA+ Aβ(17-42)	0.039
7	Sc DNA+Aβ(1-40)	0.017
8	Sc DNA+ Aβ(1-42)	0.036
9	Sc DNA+ Aβ(1-43)	0.0105

2.3.6. Melting temperature of ScDNA interacted with different fragments of Aβ

Melting temperature studies indicate the stability of DNA. The denaturation of ScDNA takes place in two steps. (i) Uncoiling of ScDNA into linear double stranded (ds) DNA and (ii) separation of linear dsDNA into single stranded (ss) DNA. The melting temperature studies (Tm) showed that ScDNA has biphasic Tm ($Tm_{1=}$ 62^0 C and Tm_2 =83^0C). There is a decrease in both Tm1 and Tm2 of ScDNA when it was interacted with Aβ(1-28), Aβ(2-35) and Aβ(1-42) (fig2.3.6). But there is an increase in Tm1 and decrease in Tm2 of ScDNA interacted with Aβ(22-35), Aβ(1-11), MAβ(1-11), Aβ(17-42) and Aβ(1-40). But in case of ScDNA interacted with Aβ(1-43) there is an increase in Tm1 and no change in Tm2 (Table 2.2).

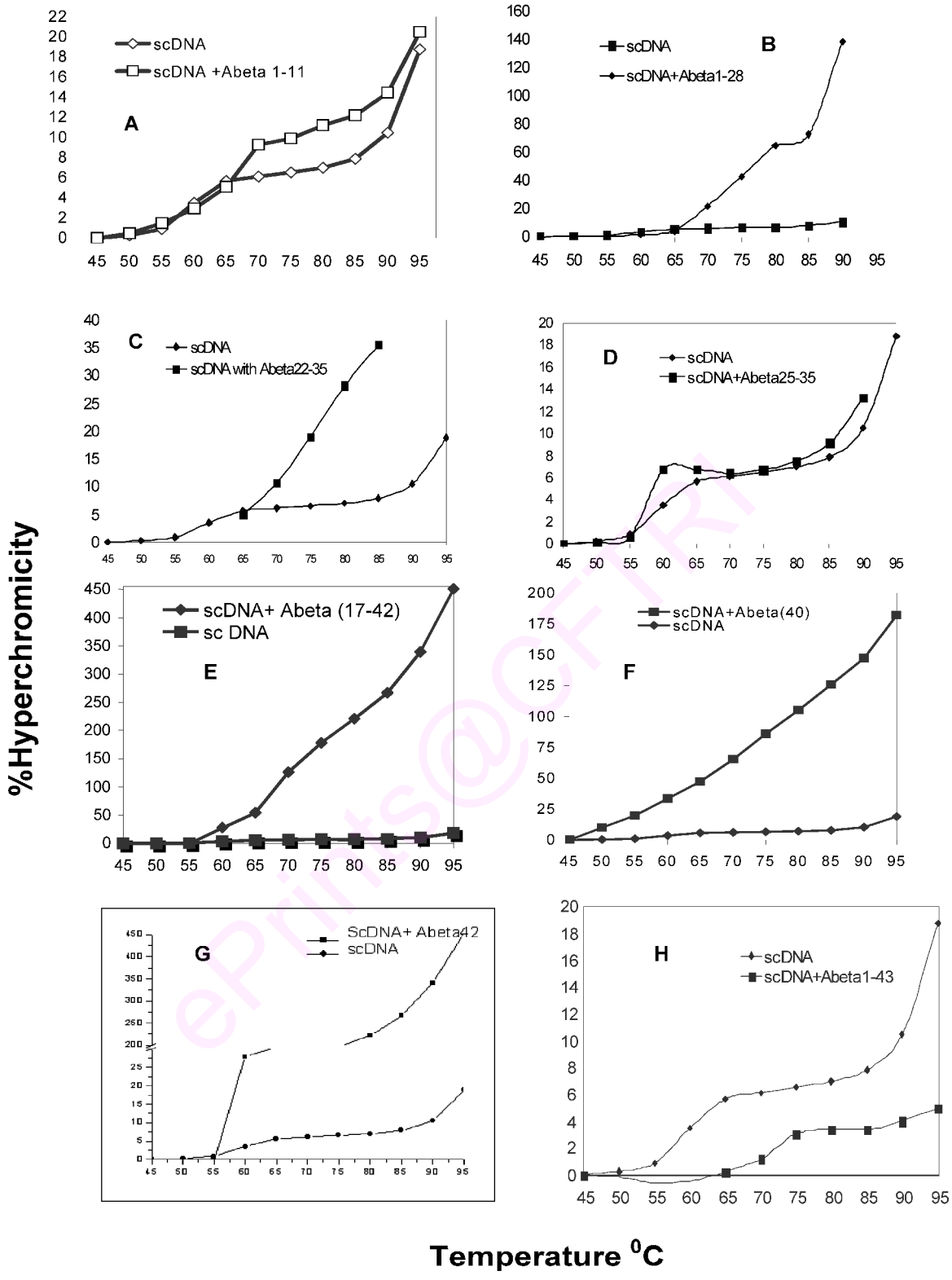


Fig 2.3.6. Melting temperature and hyperchomicity profiles of ScDNA treated with different fragments of Aβ: Heating the DNA leads to denaturation of DNA resulting to the formation of single strands. Upon denaturation of DNA, there is increase in absorbance at 260nm.

Table 2.2: Melting temperature of ScDNA in presence of different length Aβ fragments

Sl.No.	Aβ fragment and ScDNA	Tm1	Tm2
1	ScDNA alone	$62^{o}C \pm 0.47$	$83\ ^{o}C \pm 0.40$
2	ScDNA+ Aβ(1-28)	$60.5\ ^{0}C \pm 0.70$	$80.5\ ^{o}C \pm 0.40$
3	ScDNA+ Aβ(25-35)	$58.5\ ^{0}C \pm 0.40$	$81.5\ ^{o}C \pm 0.40$
4	ScDNA+ Aβ(22-35)	$69^{0}C \pm 0.40$	$81\ ^{o}C \pm 0.40$
5	ScDNA+ Aβ(1-11)	$67\ ^{o}C \pm 0.40$	$81\ ^{o}C \pm 0.40$
6	ScDNA+ Aβ(17-42)	$62^{0}C \pm 0.81$	$82.5^{0}C \pm 0.40$
7	ScDNA+ Aβ(1-40)	$79.5^{0}C \pm 0.40$	$81.5\ ^{o}C \pm 0.40$
8	ScDNA+ Aβ(1-42)	$55^{0}C \pm 0.40$	$80^{0}C \pm 1.0$
9	ScDNA+ Aβ(1-43)	$71^{0}C \pm 0.70$	$83\ ^{0}C \pm 0.40$

2.3.7. Effect of Aβ(1-11) and M Aβ(1-11) on conformation of ScDNA using CD

CD indicates the secondary conformation of DNA. To know the effect of Aβ(1-11) and M Aβ(1-11) on the conformation of ScDNA, we have interacted Aβ(1-11) and M Aβ(1-11) with ScDNA for 12h at room temperature. The samples were analyzed using circular dichoism spectroscopy. The results showed that there is a slight shift in the peak at 220nm from negative to positive in case of DNA interacted with both Aβ(1-11) and M Aβ(1-11) compared to ScDNA alone. Also, there is an increase in the 280nm positive peak of ScDNA interacted with M Aβ(1-11)(fig 2.3.7).

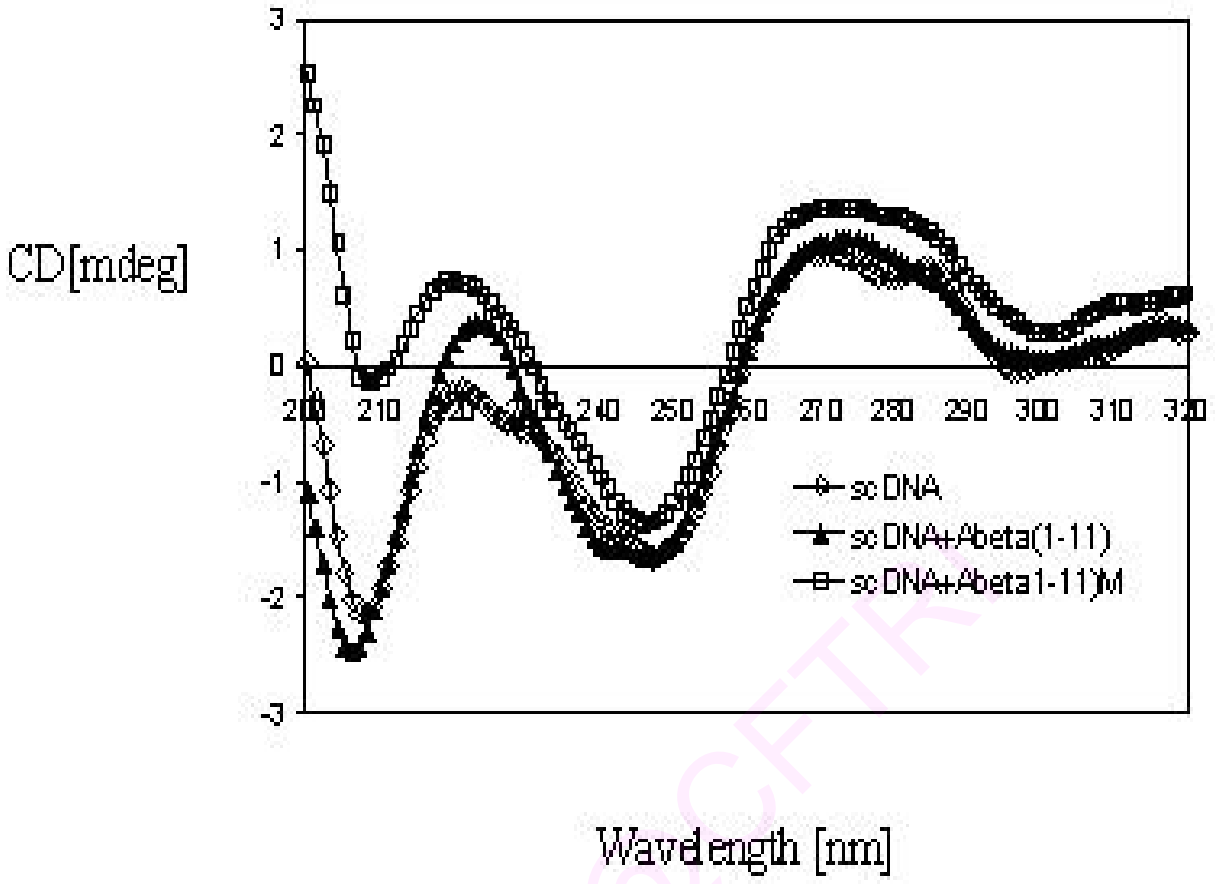
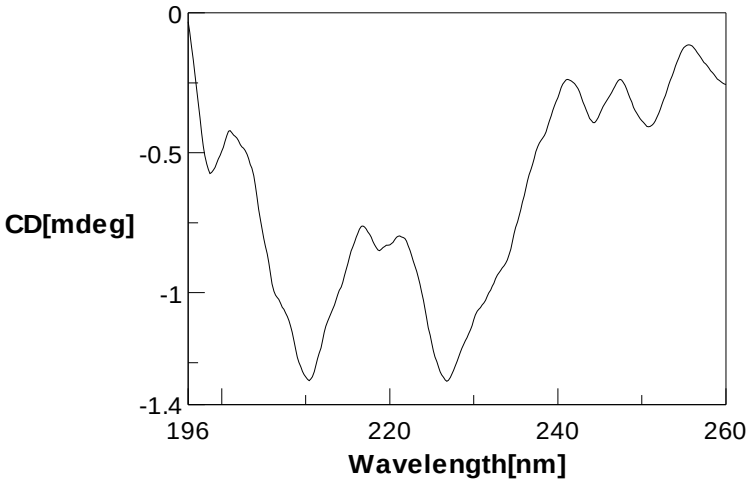


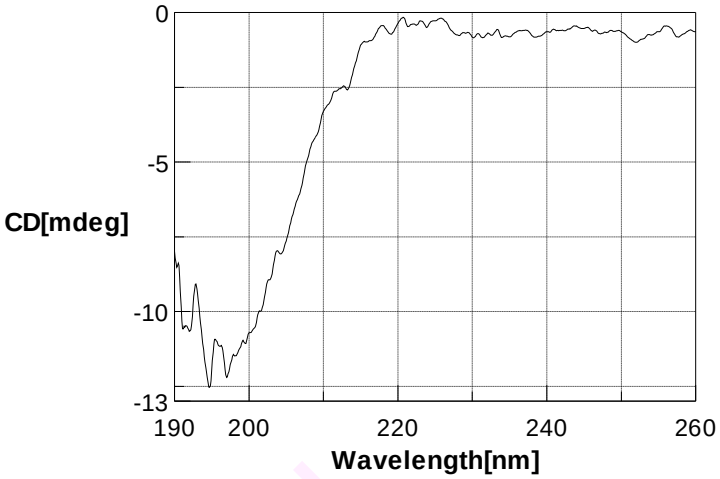
Fig 2.3.7: Effect of modified Aβ(1-11) and MAβ(1-11) on the conformation of ScDNA: 15µg ScDNA was incubated with 50µM of Aβ(1-11) and MAβ(1-11) in 10mM of Tris-Cl (pH.7.4) for 12h. The sample was analyzed for change in the DNA conformation using CD spectroscope. Aβ(1-11) and MAβ(1-11) converted negative peak to positive peak at 220nm and reduction in the 280nm peak.

2.3.8.Circular dichoism spectroscopic study of different fragments of Aβ[1-11, 1-28, 22-35, 25-35 and 1-43]

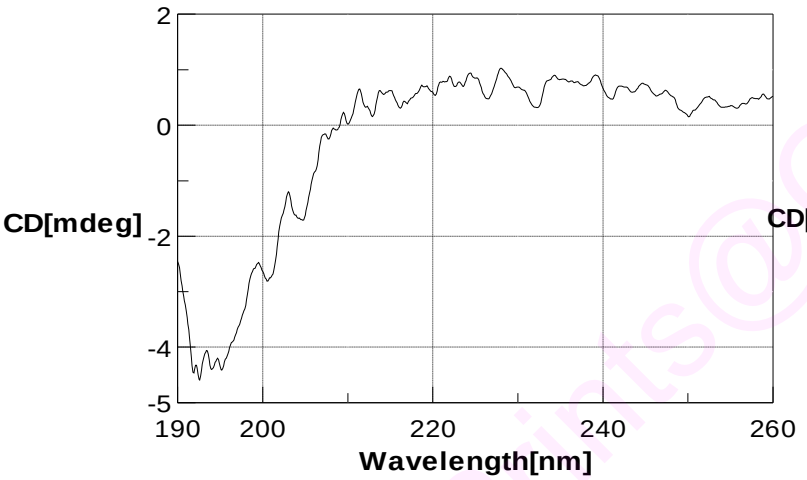
50µM of different fragments of Aβ[1-11, 1-28, 22-35, 25-35 and 1-43] incubated with for 12 h at 37 ^{O}C in 10mM Tris-Cl (pH7.4). The secondary conformation of different fragments of Aβ was recorded on JASCO J 700 Spectropolarimeter at 25 ^{O}C, with 2mm cell length with wavelength scan between 195 and 260nm. The results showed that all the fragments are in random coil conformation (Fig 2.3.8).



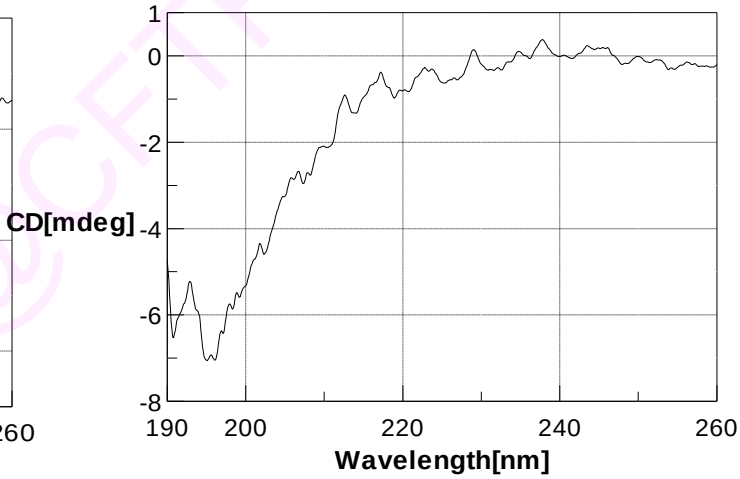
Aβ(1-11)



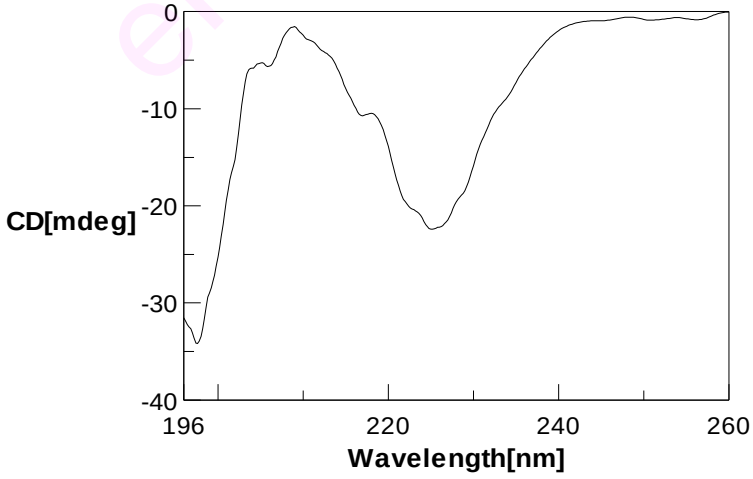
Aβ (1-28)



Aβ (22-35)



Aβ (25-35)

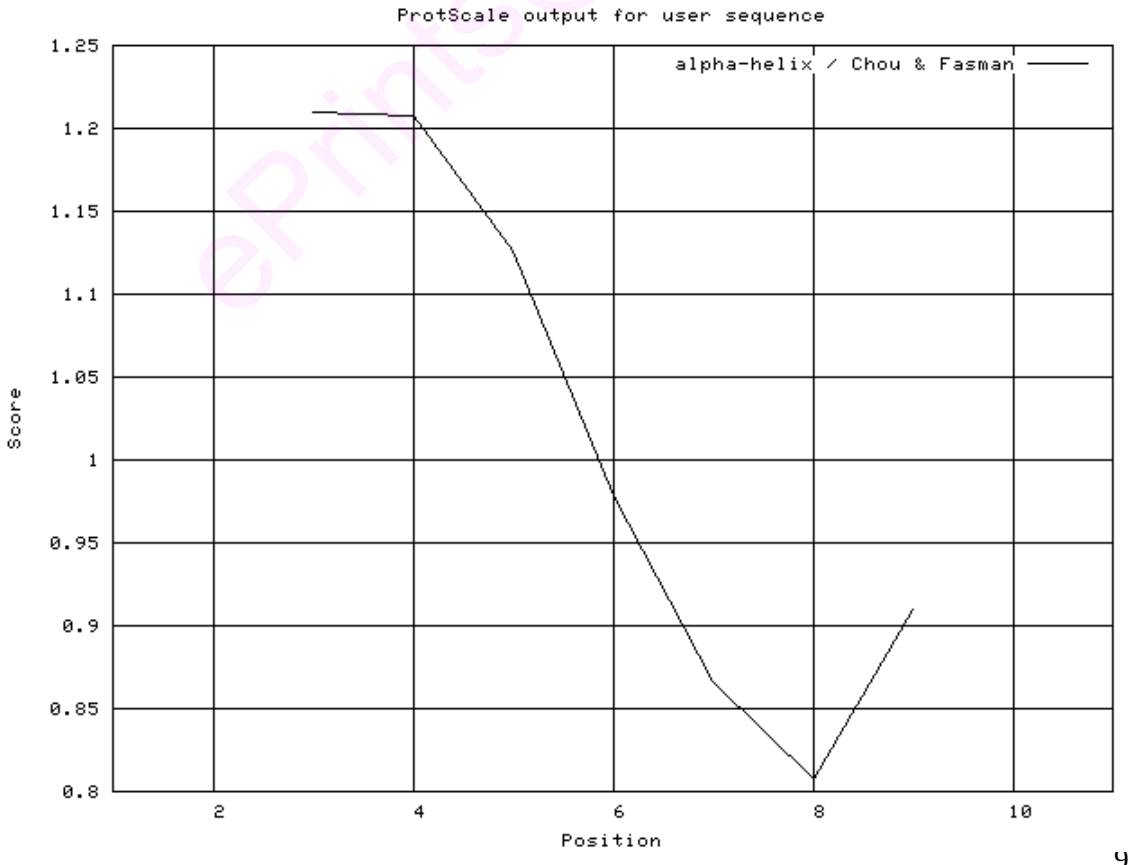


Aβ (1-43)

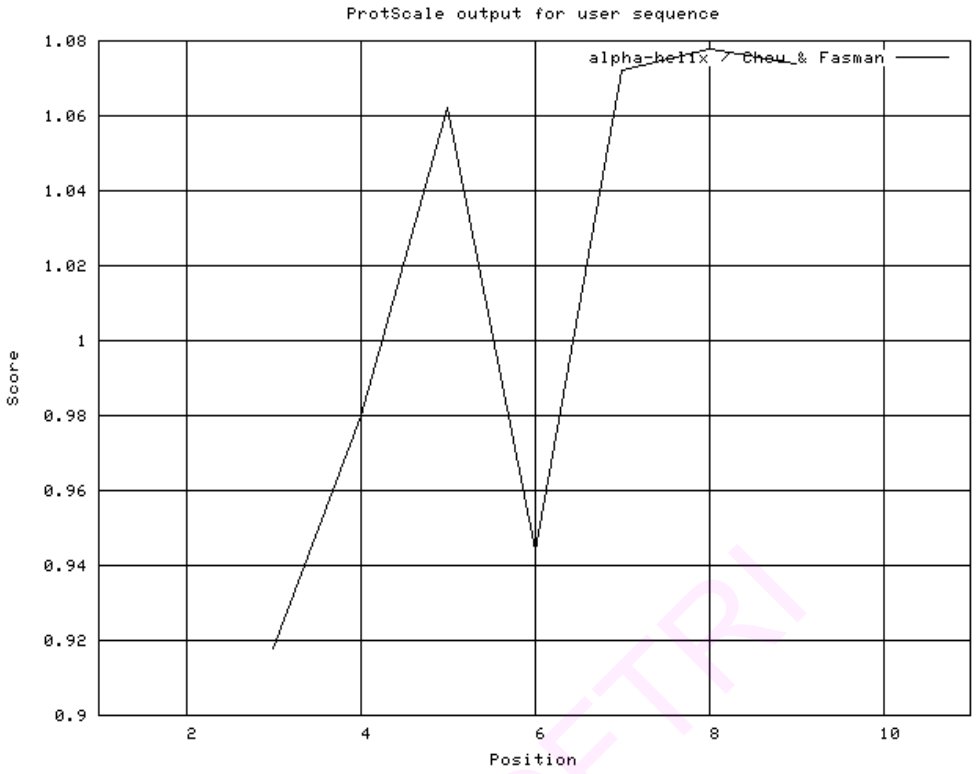
Fig 2.3.8. Secondary conformation of Aβ fragments (1-11, 1-28, 22-35, 25-35 and 1-43).The analysis of secondary conformation was done using CD spectroscopy. All the fragments analyzed is in random coil conformation.

2.3.9.Protscale analysis of secondary conformation of Aβ[(1-43, 1-42, 1-11, 25-35)]

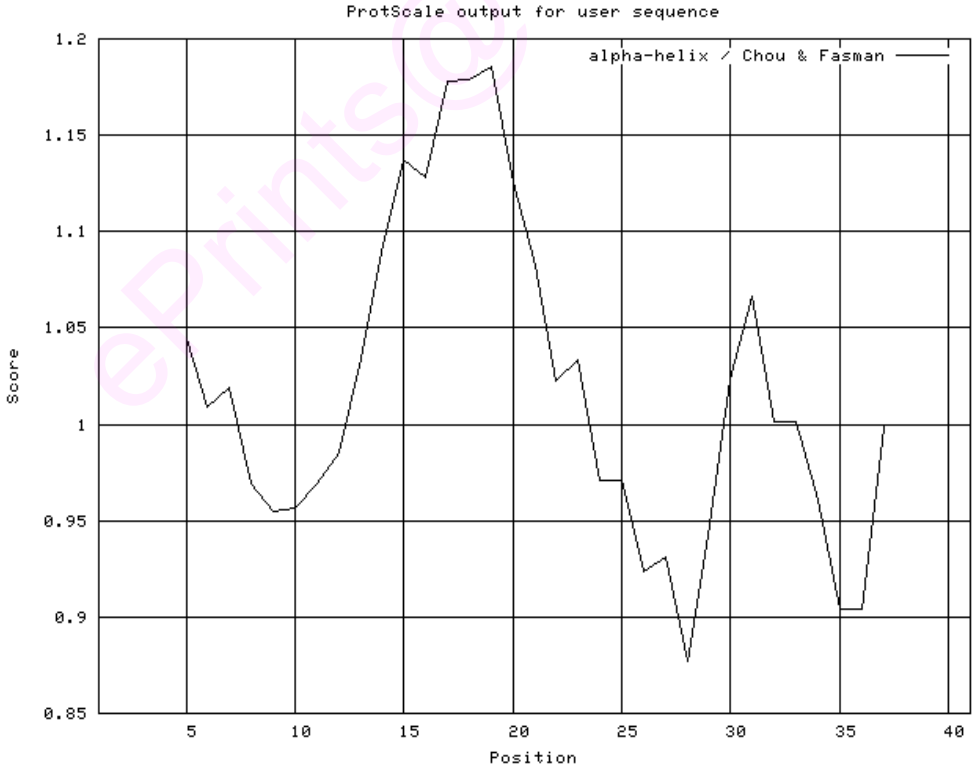
The Protscale analysis of Aβ[(1-11, 25-35, 1-42 and 1-43)] was done as they are biologically active and present in the human brain of SAD and FAD [Miravalle *et al*., 2005; Van Vickle *et al*., 2008]. The protscale was used to elucidate to understand the extent of the contribution of different amino acid residues to protein conformation. The amino acid sequences of Aβ[(1-11, 25-35, 1-42 and 1-43)] were subjected to protscale input. The protscale analysis is represented in the following graphs. The X-axis represents the sequence of amino acid residues and the Y- represents the score (extent of contribution to particular protein conformation). The amino acids such as lysine, leucine and glycine contribute to α- helix. The amino acids such as alanine, histidine and methionine contributes more to β- sheet (Fig 2.3.9).



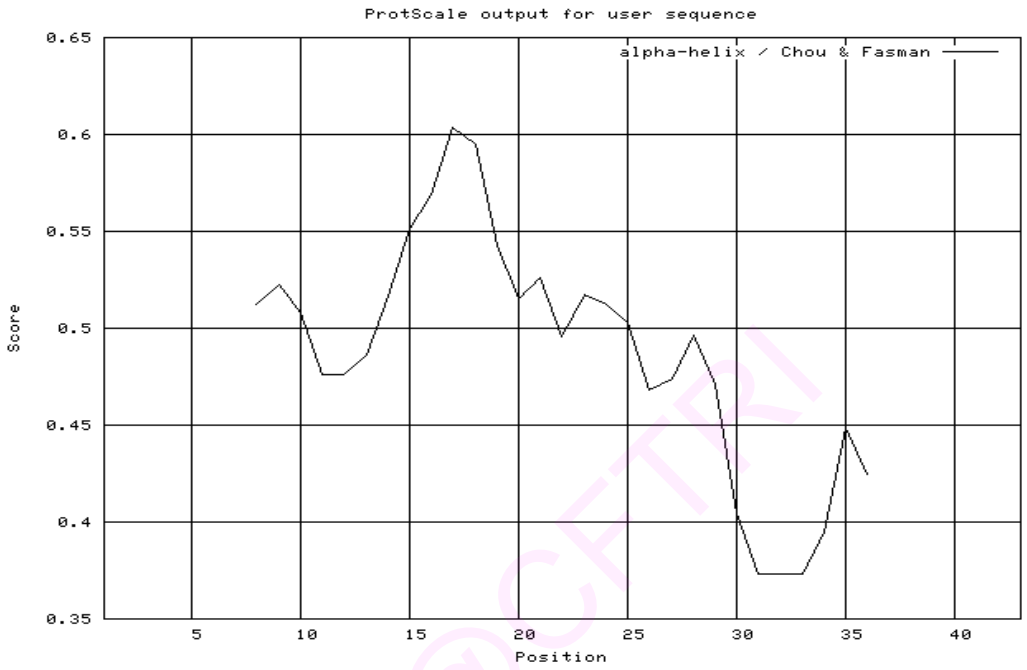
Aβ (1-11)



Aβ (25-35)



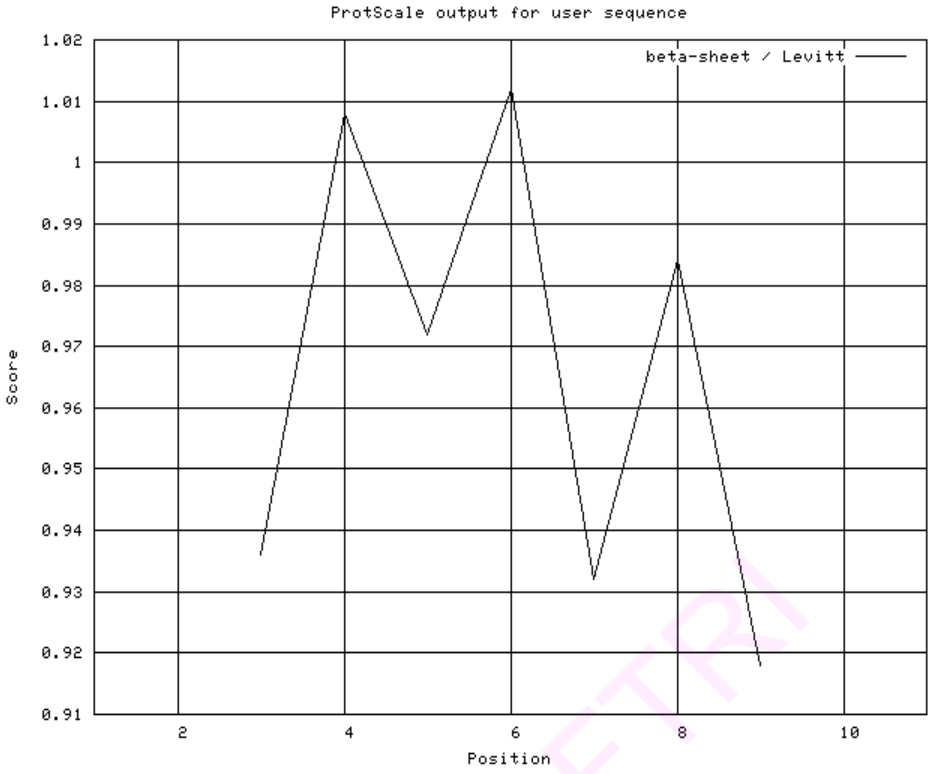
Aβ(1-42)



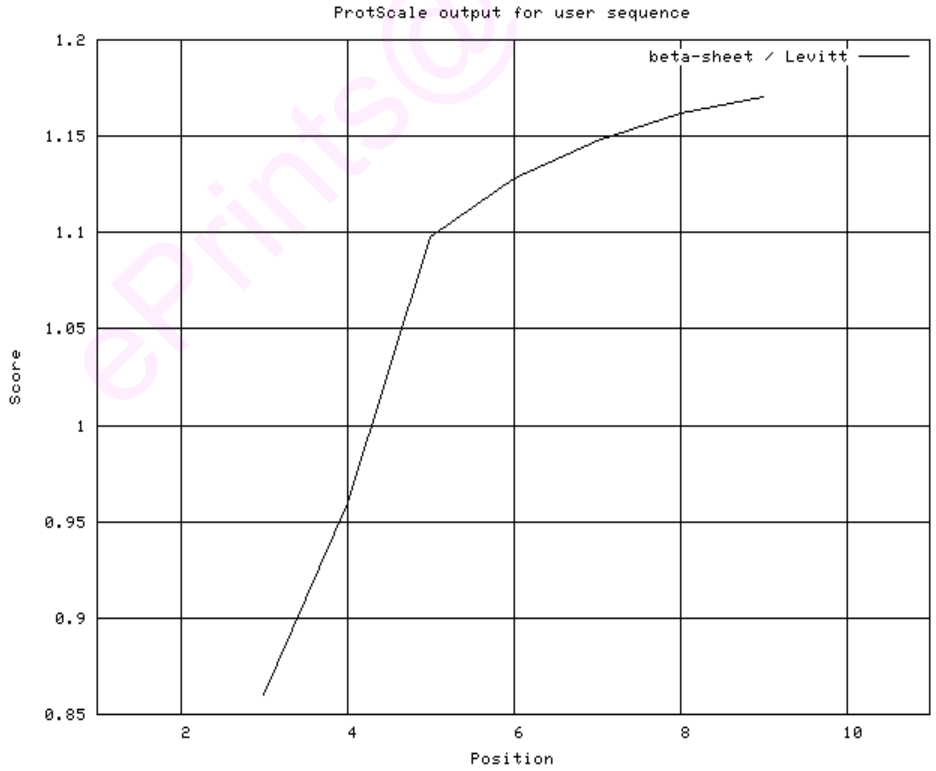
Aβ (1-43)

DAEFRHDSGYEVHHQKLVFAEDVGSNKGAIIGLMVGGVGIAT-(Aβ1-43)

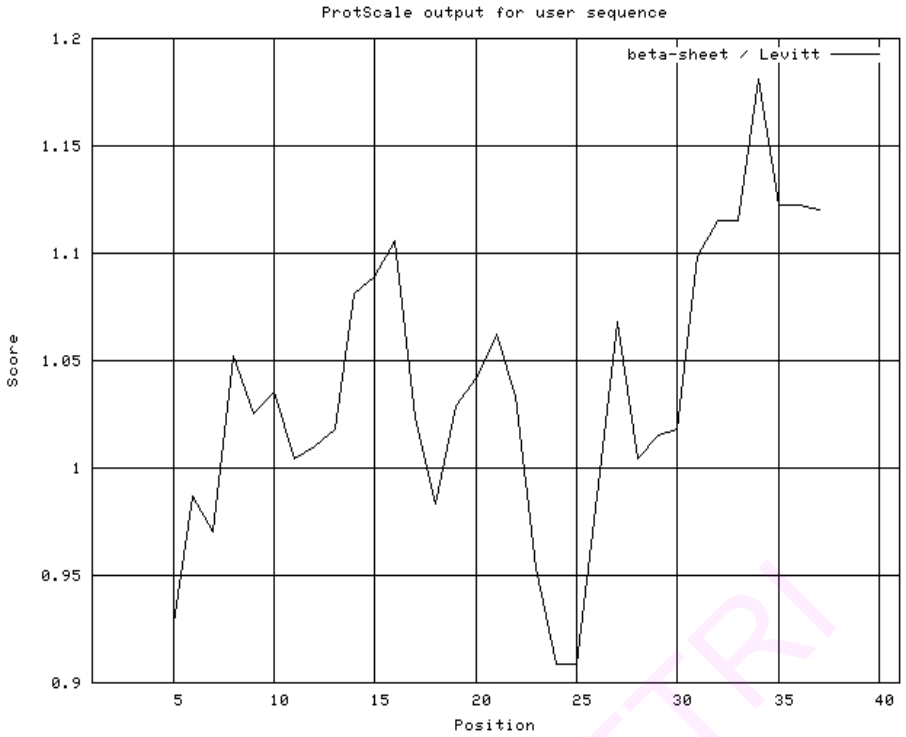
Fig 2.3.9. Prediction of extent of contribution of each amino acid in Aβ[(1-11, 25-35, 1-42 and 1-43)] to α-helix.



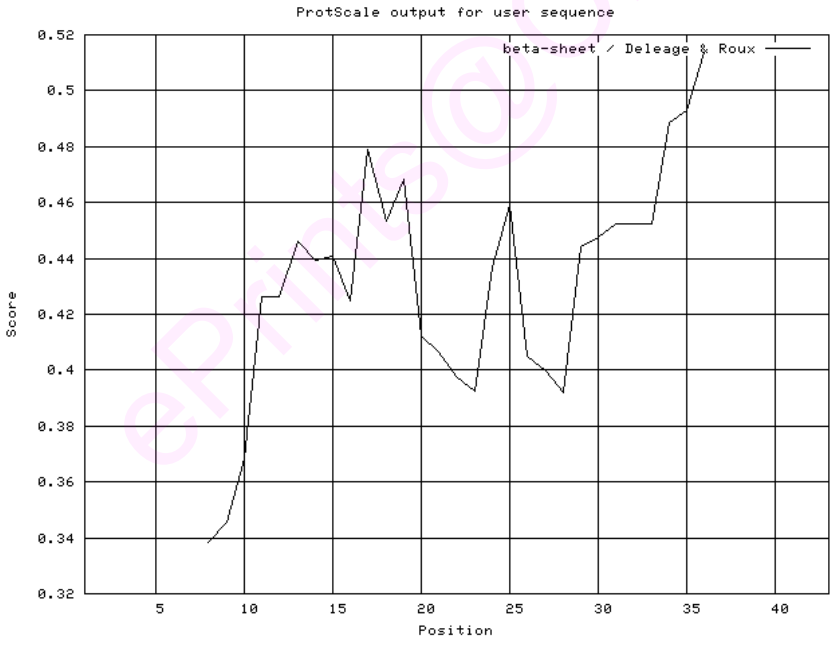
Aß(1-11)



Aβ(25-35)



Aβ(1-42)



Aβ(1-43)

DAEFRHDSGYEVHHQKLVFAEDVGSNKGAIIGLMVGGVGIAT-(Aβ1-43)

Fig 2.3.10. Prediction of extent of contribution of each amino acid in Aβ[(1-11, 25-35, 1-42, 1-43)] to β-sheet.

2.4.Discussion

AD is associated with loss of neurons that leads to behavioral problems including memory and cognition loss [Selkoe *et al*., 2001]. The intensive studies at genetic, molecular, biochemical level suggests relevance of amyloid hypothesis in AD [Tanzi and Bertram, 2005]. The proteases in AD brain known to truncate the racemized Aβ (40) to more toxic Aβ (25-35) in *vivo* [Kubo *et al.,* 2002]. In the case of Down syndrome, the N-terminal fragment of Aβ, Aβ (1-28) has been reported be present [Gyure *et al*., 2001]. The Down syndrome patients can develop AD in 40 years [Gyure *et al*., 2001]. This suggests the existence of truncated small peptides in the AD brain [Kaminsky *et al*., 2010]. The Aβ(25-35) also found in different cellular compartments such as mitochondria, cytosol, lysosomes and nucleus [Kaminsky *et al*., 2010]. This suggest the use of small peptides [Aβ(1-11), Aβ(1-28), Aβ(22-35) Aβ(25-35)] derived from Aβ (1-42) makes relevance in studying the genotoxicity of these peptides.

Amyloid hypothesis suggests that Aβ accumulates as the initial pathologic factor for the development of AD [Hardy and Higgins, 1992]. Aβ is a prime factor implicated in the cause of AD [Selkoe, 2001]. Our lab for the first time showed that Aβ(1-42) binds and relaxes ScDNA to both open circular and linear form [Hegde *et al*., 2004]. Also, Suram *et al*. [2007] reported that Aβ(1-42) nicks the ScDNA. The present study is undertaken to understand mechanism of Aβ induced DNA nicking and which fragment of Aβ [N-terminal or C-terminal of Aβ(1-42)] involved in nicking the ScDNA. We found that N-terminal fragments of Aβ, Aβ (1-11), Aβ(1-28), Aβ(1-40), and Aβ(1-42) nicks ScDNA and causes the formation of both linear and open circular forms. To prove our hypothesis that histidine is involved in nicking DNA, we modified histidine at 11^{th} position in Aβ(1-11). The histidine modified Aβ(1-11) did not nick ScDNA. To show whether, the DNA nicking activity of Aβ peptides is specific to ScDNA, different forms of DNA, such as ScDNA, linear double stranded λDNA, single stranded circular and double stranded DNA were treated with Aβ(1-11)/ Aβ(1-11) M for 12 h at room temperature (Fig 2.3.2). The synergic interaction

of genetic and environmental factors leads to the AD pathology. Aβ may cause genotoxicty in the AD patients [Suram *et al.,* 2006]. The previous reports from our lab and others have showed that Aβ localizes in the nucleus and found near to DNA [Gouras *et al*., 2000; Grant *et al*., 2000; Hegde *et al*., 2004]. Additionally, Aβ(1-42) also induced the conformation of B-form of ScDNA to ψ-DNA, which is closely associated with Z-DNA [Hegde *et al*., 2004]. Suram *et al*. [2007] showed that Aβ(1-42) damages the ScDNA by nicking. Recently, Ramesh *et al*. [2010] showed that Aβ(1-42) alters the DNA conformation from B-DNA to C-type, π- and ψ-DNA in frontal cortex, hippocampus and midbrain respectively in aged rabbit. Additionally, Aβ(1-42) also caused the DNA instability in aged rabbit brain. Aβ is a metalloprotein and it binds to transition metals such as iron and copper and zinc [Huang *et al.,* 1999]. The ScDNA nicking activity of Aβ(1-42) is modulated by binding of Mg^{2+} and Ca^{2+} [Suram *et al.,* 2007]. The Mg^{2+} enhanced the ScDNA nicking activity, of Aβ(1-11), Aβ(1-28), Aβ(1-40) and Aβ(1-42). Surprisingly, in presence of Mg^{2+}, Aβ(22-35), Aβ(25-35), Aβ(17-42) and Aβ(1-43) nicked the ScDNA and caused the formation of both open and linear forms. Modification of histidine had abolished the metal binding ability to Aβ [Atwood *et al*., 1998]. We hypothesized the involvement of histidine in nicking the ScDNA by Aβ. ATA is a potent inhibitor of most nucleic acid binding enzymes. It competes with protein for nucleotide bases for its binding [Hallick *et al*.,1977]. The modified Aβ(1-11) (histidine at 11^{th} position) did not nick ScDNA. We have used ATA to inhibit the nicking activity of different fragments of Aβ in presence of Mg^{2+}. ATA is found to inhibit the nicking activity of Aβ(1-42) only and it did not inhibit the ScDNA nicking activity of Aβ(1-11), Aβ(1-28), Aβ(22-35), Aβ(25-35), Aβ(17-42), Aβ(1-40) and Aβ(1-43). So, from the above results we opine that different fragments of Aβ follow different mechanism in nicking the ScDNA.

Further, we have studied the effect of different fragments of Aβ on stability of ScDNA by Tm and Etbr binding. The melting temperature studies will provide information on the stability of DNA. The higher the Tm greater the stability of DNA. The ScDNA will follow a pattern having two phases in denaturing the ScDNA. First Tm1 is corresponds to uncoiling ScDNA and Tm2 corresponds to denaturing the uncoiled ScDNA. If there is any changes in the above-mentioned pattern it may be

attributed to interaction of Aβ with ScDNA. The interaction studies on ScDNA with different Aβ fragments showed varied Tm response curves and the corresponding changes in the Tm values (Table 2.2). The earlier report suggests that Aβ(1-42) retained two phase pattern of ScDNA with two Tm values (Tm1-59 ^{0}C, Tm2- 88 ^{0}C) but Aβ(1-16) did not alter the Tm [Hegde *et al* ., 2004]. Our Tm and EtBr binding studies suggests that Aβ(1-11), Aβ(1-28, Aβ(1-40) and Aβ(1-42) decreased the stability of ScDNA which reflected in decrease in Tm and increase in the EtBr binding, which is further supported by agarose gel study results. While, Aβ(17-42), Aβ(22-35), Aβ(25-35) and Aβ(1-43) though decreased the Tm but these results are not supported by agarose gel study. Additionally, EtBr binding to ScDNA indicate the intercalation between the two strands of the DNA. The EtBr binding studies suggests that there is a linearization of the ScDNA and results in increased EtBr binding. Again, these results are not supported by the agarose gel study results.

Circular dichoism data will give the information on the global conformation of the peptides, or protein and DNA. The histidine modified Aβ (1-11) was interacted with ScDNA and analyzed DNA conformation. The modified Aβ (1-11) and unmodified Aβ (1-11) could shift peak at 220nm from negative to positive compared to ScDNA alone. This data further confirms that Aβ (1-11) interact with DNA rather directly. The role of conformation of Aβ may not be ruled on DNA nicking property of Aβ. The organic molecules such as trifluroethanol (TFE) is known to convert random coil conformation of Aβ to α-helix [Barrow *et al*., 1992] and α-helix is safe conformation of Amyloids [Barrow *et al*., 1992]. The instability of the genome in all the organisms can be induced by exogenous and endogenous agents that induces DNA lesions, DNA breaks etc. [Bohr *et al*., 2007]. The brain cells which are non-replicating are particularly vulnerable to DNA damage since DNA damage accumulation leads to deleterious effect on neuronal cells [Boh *et al*., 2007]. The studies involving patients with AD, PD, HD, ALS, XP suggests that oxidative stress and DNA damage are common mechanisms in the neuronal cell death [Kraemer *et al*., 2007; Trushina and McMurray, 2007]. The chronic exposure to reactive oxygen species (ROS) may be a risk factor for neurodegenerative disorders and aging (Akbari *et al*., 2006). Hence, ROS are important genotoxic molecules that are responsible DNA damage. ROS are

known to cause DNA lesions which includes DNA single and double strand breaks, oxidized DNA bases and repaired by base excision repair (BER) (Seeberg *et al.,* 1995). Suram *et al*. [2006] suggests that Aβ directly causes genotoxicity by damaging the DNA. Suram *et al*. [2007] Aβ(1-42) nicks DNA and destabilize the same. Recently, Ramesh *et al*.[2010] Aβ(1-42) causes the DNA instable and changes the DNA conformation in aged rabbit brain. This study will open the new line of thought on the role of Aβ in the neurodegenerative process of AD. In conclusion our studies give data on the differential effects of different fragments of Aβ on the DNA stability. The modification of histidine in Aβ (1-11) did not nick the ScDNA, indicating the role of histidine in nicking the ScDNA. Mg^{2+} has enhanced the nicking activity of Aβ peptides. This data indicates that amino acid sequences play a significant role in guiding Aβ DNA nicking activity.

Chapter 3A-a

Aβ(1-42) induced genomic instability in aged rabbit brain

Chapter 3A-a Aβ(1-42) induced genomic instability in aged rabbit brain

3A-a.1. Introduction

DNA instability has been implicated in the pathogenesis of neurodegenerative disorders including Alzheimer's disease, amyotrophic lateral sclerosis, Down's syndrome, Parkinson's disease, etc. (Kraemer *et al*., 2007; Bhor *et al*., 2007). The data on DNA damage indicates that the damage is associated with disease as a pathological event, however the role of DNA damage as a major event in neurodegeneration is still not clear (Bhor *et al*., 2007). Amyloid accumulation in the form of senile plaques is well known in AD brain. Amyloid is hypothesized to cause genotoxicity leading to neuronal cell death (Suram *et al*., 2006). There is a new evidence that amyloid induces genomic instability and also alter the DNA topology *in vitro* (Hegde *et al*., 2004). Recent studies from our lab and elsewhere showed the nuclear localization of Aβ(Gouras *et al*., 2000; Grant *et al*., 2000; Suram *et al*., 2007) and its DNA binding ability (Hegde *et al*., 2004). Suram *et al*. (2007) reported that Aβ induces DNA damage by nicking like endonucleases. Ghribi *et al*. (2001) showed that intracisternal injection of Aβ(1-42) in aged rabbits, causes the induction of genes coding for gadd153. This gene is involved in growth arrest and DNA damage. Previous studies showed that the magnitude of gadd153 expression appears to be proportional to the extent of DNA damage (Price and Calderwood, 1992). Studies have shown that there is a positive correlation between the Aβ load and atrophy in AD patients (Hardy and Higgins, 1992; Anderson *et al*., 2000). All these studies insight, the potential role of Aβ(1-42) in causing neuronal cell death, but the mechanism is still not clear. Further, the role of Aβ(1-42) in causing neurodegeneration through genomic instability is unexplored area. The present study aims to answer three specific questions, (a) Does Aβ(1-42) injection to aged rabbits alter DNA stability?, (b) Does Aβ(1-42) alter the DNA conformation? and (c) Does Aβ(1-42) alter the structural changes in the aged rabbit brain? These questions may likely to answer the possible role of Ab in neurodegeneration through genomic instability.

3A-a.2. Material and methods

Aβ(1-42) was purchased from rPeptide, USA. Phenol, chloroform, isoamyl alcohol, sodium chloride, SDS, Tris, HEPES, EDTA sodium salt, glacial acetic acid, ethanol, sodium hydroxide, bromocresol green, and ethidium bromide were purchased from SRL Pvt. Ltd. DNase I, RNase, proteinse K, DNA marker (1 kb) and loading dye were purchased from Bangalore Genei, Bangalore, India.

3A-a.2.1. Animals and treatment protocol

All animal procedures were carried out in accordance with Indian National Science Academy (India) animal protocol guidelines and rules framed by J.S.S. Animal Ethical Committee, J.S.S. Medical College, and Mysore, India. All the animals were housed in JSS Animal house in separate stainless steel cages. Six New Zealand white aged rabbits (4.0 yr old) received intracisternal injections of 100 mL of normal saline (control) and another batch of six aged rabbits received 100 mL of (1 mg/mL) Aβ(1-42) in saline. The injections were carried out under calmpose anesthesia, according to the protocol described previously (Savory *et al*., 1999). All the aged rabbits were euthanized after 25 days, after the Aβ(1-42) injected animals have developed neurological symptoms including forward head tilting, hemiplegic gait, loss of appetite, isolation behavior, splaying of extremities and paralysis. These behavioral changes were the result observations by neurologist and Psychiatrist. Both the clinicians assigned arbitrary values for behavioral changes. There was 30% forward head tilting and hemiplegic's gait, while 40% loss of appetite as evidenced by food uptake, 50% isolation behavior, 25% splaying of extremities and paralysis. Further in-depth analysis on the tests related to memory and cognition are in progress with long-term studies. The control animals did not display any behavioral disturbances. The MRI was done for both rabbits before Aβ(1-42)/saline injection and 25 days after Aβ(1-42)/saline injection. The animals were sacrificed and brains were removed and different regions were separated and then stored at -80^{0}C. The different regions dissected include FC, H and M. The tissue was used for isolating genomic DNA. The genomic DNA was isolated using standard protocol with modifications as described below (Hegde *et al*., 2006).

3A-a.2.2. Isolation of genomic DNA from rabbit brain tissue

Brain tissue was cut into small pieces and was transferred into an autoclaved porcelain mortar and pestle. All glasswares, mortar, pestle, etc., were autoclaved to avoid bacterial contamination. Liquid nitrogen was poured into the mortar and the tissue was allowed to freeze. Tissue was ground thoroughly with pestle with frequent additions of liquid nitrogen. Sufficient quantity of liquid nitrogen was poured into the mortar and was swirled. Tissue homogenate was transferred into a sterile tube and the liquid N2 was allowed to evaporate (a sterile spatula was used to transfer the powdered tissue into a graduated tube). The tissue homogenate was incubated with lysis buffer (50 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 100 mM NaCl) and was added with 15 mg/mL of proteinase K and 2% SDS in the final volume. One milliliter of lysis buffer was used for every 500 mg of tissue. (Note 1: Lysis buffer should be pre-warmed, Note 2: Add proteinase K after first 2 h, optimum: 3 h.) The homogenate was incubated at 37 ^{0}C in a water bath for 12–16h or over night. After the completion of incubation, the incubated lysate was transferred to an autoclaved 50 mL conical flask. Then, equal volume of Tris-saturated phenol (pH 8.0) was added and mixed thoroughly, either manually or mechanically for 10 min. The lysate was centrifuged for 10 min at 10,000 rpm at 13 ^{0}C. The supernatant was collected into a fresh autoclaved 50 mL conical flasks and 1/2 volume of Tris-saturated phenol and chloroform: isoamyl alcohol was added and mixed thoroughly. One part phenol: one part chloroform (C) and iso-amyl alcohol (IA) mixture (C:IA = 23:1). (Note: Tris saturated phenol was stored in amber colored bottles at low temperature to avoid oxidation of phenol.) The supernatant and Tris-saturated phenol–chloroform mixture was centrifuged at 5000 rpm at 4 ^{0}C. The upper aqueous layer was collected into a fresh tube and 1/30th volume of sodium acetate (pH 5.5) and equal volume of chilled absolute ethanol was added. DNA was precipitated by slowly swirling the tube manually. (Note: Pre-cooled tubes were used and DNA was transferred into another tube containing 70% alcohol for washing). DNA was washed twice with 70% alcohol and once with absolute alcohol to remove excess salt and vacuum dried and Stored at –20 ^{0}C. The vacuum dried DNA was dissolved in 1 mL of TE buffer (10 mM Tris–HCL, 1 mM EDTA, pH 8.0). The DNA isolated from cells also contains RNA, which was removed by digesting the preparation with RNase enzyme. RNase solution was kept in boiling water for 10 min before use so as to inactivate DNase I. The method

provides high quality genomic DNA with good yield. It is important to mention that the genomic DNA was isolated from total brain tissue of different brain regions (i.e., containing both neurons and glia).

3A-a.2.3. Precautions taken in order to prevent *in vitro* DNA damage during phenol -chloroform genomic DNA isolation

All the materials used for isolating DNA were autoclaved. Powder free gloves were used to prevent nuclease contamination from the surface of the skin. The sterile, disposable plastic ware was used. Pipettes were wiped with DNase I-removal solution (diethylpyrocarbonate). Pipette tips with blunt ends were used to prevent DNA fragmentation. Liquid nitrogen was used to keep the frozen tissue hard before grinding. This step will prevent activation of endonucleases.

3A-a.2.4. DNA purity

The concentration of DNA was measured by recording absorbance at 260 nm and purity of DNA preparation was checked by recording the ratio of absorbance at 260 nm/280 nm, the ratio needs to be 1.7. This indicates good quality of DNA. The DNA helicity and stability parameters were analyzed using established standard protocol (Hegde *et al*., 2006; Sutherland and Shih, 1983).

3A-a.2.5. DNA stability

3A-a.2.5.1. Neutral agarose gel electrophoresis

The DNA damage is assessed by running neutral and alkaline gel electrophoresis. Neutral agarose gel electrophoresis was done to analyze the DNA damage. The DNA was electrophoresed on 1.5% agarose gels in Tris–acetate–EDTA buffer (pH 8.0) at4 V/cmfor4 h. One microgram of DNA was loaded in each well. The gel was stained with EtBr in cold water for one hour and photographed in UV gel documentation system (Hero labs). DNA ladder (1 kb) was used as a molecular marker.

3A-a.2.5.2. Melting temperature of the genomic DNA

DNA was dissolved in HEPES buffer, 0.01 M (pH 7.4). The DNA was used at a concentration of 12.5 mg/mL. The melting profiles (Tm) of DNA were recorded in

Spectrophotometer (Ultraspec, 4300 probe) equipped with thermo-programmer and data processor (Amersham pharmaciabiotech, Hong Kong). The temperature point at which there is 50% hyperchromic shift is taken as Tm of the DNA sample. Tm values were determined graphically from the hyperchromicity vs. temperature plots. The experiments were repeated thrice to validate the results.

3A-a.2.5.3. Ethidium bromide binding studies

The quantification of ethidium bromide (EtBr) bound in moles per base of genomic DNA was measured in 0.01 M Tris–Cl (pH 7.4) using HITACH F-2000 Fluorescence Spectrophotometer. The fluorescence was measured by titrating constant amount of DNA with increasing EtBr against the blank containing no DNA. The measurements were performed keeping excitation at 535 nm and emission at 600 nm with 10 mm path-length. The amount of EtBr bound per base pair of DNA was calculated using scatchard plots of 'r' vs. 'r/Cf' (Scatchard, 1949). The concentration of bound EtBr in 1.0mL dye-DNA mixture (Cb') was calculated using the equation:
$Cb'=[C_o (F-FO)/(V/Fo)]$, where

Co =Concentration of EtBr (pmoles) in the dye complex mixture

F= Observed fluorescence of EtBr at any point of dye-DNA mixture

Fo= Observed fluorescence of EtBr with no DNA

V=Experimental value, ratio of bound EtBr to free EtBr at saturation point.

The concentration of free dye (Cf') was then calculated by using the formula

$$Cf'=Co'-Cb'$$

Where, Cf, Co'and Cb' were expressed in pmoles. The amount of bound EtBr bound per base pair was calculated by r=Cb' (pmoles) / DNA concentration (pmoles of base pair). A plot was made for r vs. r/Cf and the point where the straight line interacts the axis r was defined as the maximum amount of dye bound per base pair (n), where $Cf=Cf'x10^5$ M.

3A-a.2.5.4. DNase I sensitivity assay for DNA stability

A saturated reaction mixture of genomic DNA and EtBr (1:1, w/w) was treated with DNase I (0.5 mg/mL) and fluorescence emission was monitored with time (0– 30 min) at excitation at 535 nm and emission at 600 nm using HITACHI F-2000 spectrofluorimeter. The fluorescence values were plotted vs. time.

3A-a.2.6. DNA conformation analysis by circular dichroism (CD) spectroscopy

The CD spectra (210–330 nm) were recorded for rabbit brain genomic DNA in 0.01 MTris–Cl buffer (pH 7.4) on JASCO 810 Spectropolarimeter. The cell length and width is 1 mm each. Each spectrum is the average of triplicate recordings. Twenty micrograms of DNA from each sample was used. The DNA conformation was characterized using reference of Gray *et al*. (1992).

3A-a.2.7. Imaging studies

The rabbit's brain was imaged using MRI before injecting the Aβ□(1-42). MRI images of aged rabbits injected Aβ□(1-42) were taken after 25 days of injection. Scanning was done on Siemens Concerto Open System 0.2 T MRI scanner. Animal head was scanned using standard head coil. All rabbits were sedated by giving 10 mg of IV Diazepam (Calmpose) through vein in rabbit's ear lobe. Animals were under the influence of diazepam for about 20 min, which was sufficient to complete the MRI protocol. Aged rabbits were scanned in prone position. Images were acquired in coronal plane using T1, T2 and 3D gradient Echo Sequences. T2 sequences were obtained by using TR 4390 ms, TE 118 ms, 256 x 256 matrix, and FOV 25 cms. T1 sequences were obtained by following protocol TR 483 ms, TE 9.3 ms, 256 ˪ 256 matrix FOV 25 cm. 3D GRE sequences were obtained by following protocol—TR 20 ms, TE 89 ms, FOV 25 cm. Image analysis was done by comparing the anatomy of control scans with Aβ(1-42) injected rabbit brain. The change in thickness of lateral ventricles, frontal lobe, H and M were computed.

3A-a.2.8. Statistical analysis

All the data obtained were statistically analyzed using Student t test and significance of differences between control and Aβ(1-42) injected groups were calculated. The statistical analysis was carried out using Microsoft Excel 2003 program.

3A-a.3. Results

3A-a.3.1. DNA stability

3A-a.3.1.1. Neutral agarose gel electrophoresis

The stability of DNA is analyzed by agarose gel electrophoresis (Fig 3A-a. 1). The DNA from FC (Fig3A-a.1B) and H (Fig 3A-a. 1D) of Aβ injected rabbits showed smears indicating DNA is damaged. But DNA from control FC (Fig 3A-a. 1A) and H (Fig 3A-a 1C) are intact. But DNA from M of Aβ injected rabbit brain, showed more migration indicating condensation of DNA (Fig 3A-a 1F). But, control M-DNA showed intact DNA (Fig 3A-a. 1E). The lane 1 is 1 kb DNA ladder.

3A-a.3.1.2. Melting temperature of genomic DNA

Melting temperature studies indicate the stability of DNA. (A) FC: Aβ (1-42) injected aged rabbit brain DNA showed 80.6 ± 10.9^0C where as control DNA showed 83.8 ± 1.9 ^{0}C ($p < 0.01$) (Fig 3A-a. 2A). (B) H: Aβ (1-42) injected DNA showed 77.6 ^{0}C ± 2.9 where as control DNA showed 82.9 ± 3.7 ^{0}C ($p < 0.01$) (Fig 3A-a. 2B). The data indicates that FC and H DNA is destabilized, due to DNA damage in Ab injected rabbits (C) M: Aβ (1-42) injected DNA showed 84.0 ± 2.6 ^{0}C, where as corresponding control DNA showed 79.0 ± 3.3 ^{0}C ($p < 0.05$) (Fig 3A-a. 2C). This data further support that DNA from M of Aβ injected-aged rabbits is in condensed form as it has higher Tm.

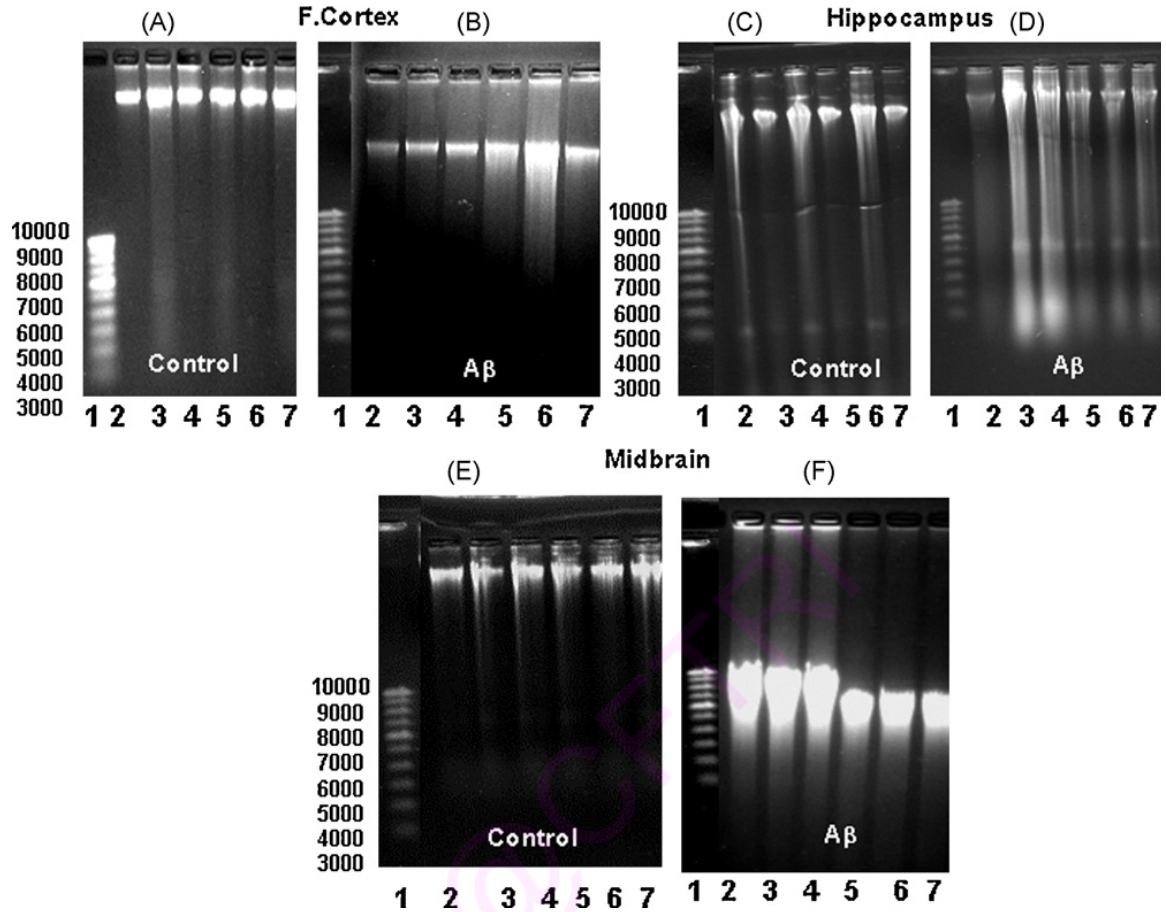


Fig. 3A-a.1. Neutral agarose gel electrophoresis pattern of genomic DNA isolated from control and Aβ (1-42) injected rabbit brains. (A) Control frontal cortex DNA; (B) Aβ (1-42) injected frontal cortex DNA; (C) control hippocampus DNA; (D) Aβ (1-42) injected hippocampus DNA; (E) control midbrain DNA; (F) Aβ(1-42) injected midbrain DNA. The lane 1 in the gel shown is 1kb marker, lanes 2–7 DNA isolated from corresponding control and Aβ (1-42) injected rabbit brain.

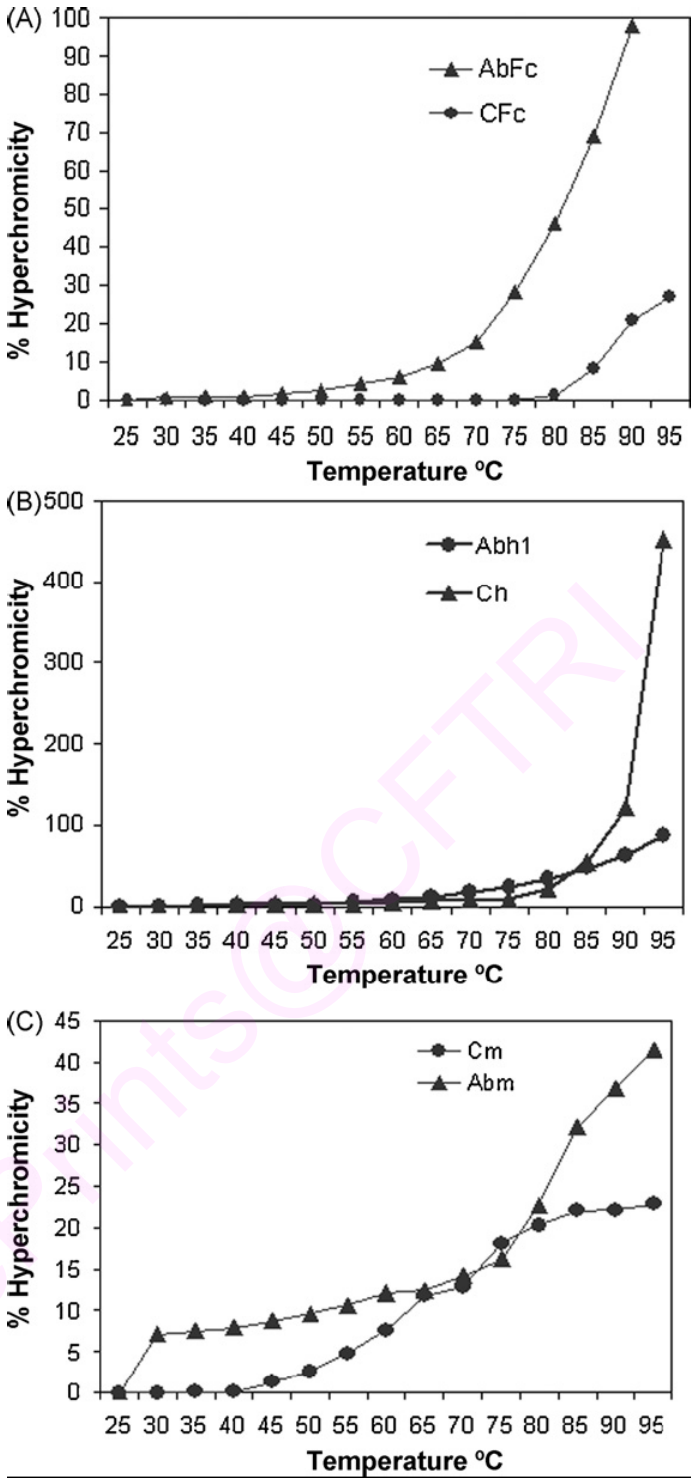


Fig. 3A-a.2. Melting temperature (Tm) of genomic DNA of different regions of Aβ (1-42) injected and control Rabbit brain. 10 mg of genomic DNA from hippocampus (h), midbrain (m), and frontal cortex (FC) of Aβ (1-42) injected and control rabbit brain was subjected to thermal denaturation. The absorbance was recorded at 260 nm form 25 to 95 8C with 1 8C rise per min using UV spectrophotometer (Amersham) with a thermostat. (A) Frontal cortex DNA; (B) Hippocampus DNA; (C) midbrain DNA. Aβ—rabbit brain injected with Aβ. C—control rabbits. FC—frontal cortex. h— hippocampus. m—midbrain.

3A-a.3.1.3. EtBr binding studies

EtBr binding studies indicate DNA stability and Scatchard plot analysis shows the number of EtBr bound per base pair (bp) of DNA (Fig 3A-a. 3). The Scatchard plot data showed that FC, H and M-DNA showed significant reductions in number of EtBr bound per base pair ($p < 0.05$) with reference to their respective controls (Fig 3A-a. A–C). The absolute data on the number of EtBr molecules bound per bp of DNA is compared in Table 3A-a.1.

3A-a.3.1.4. DNase I sensitivity assay to study DNA stability

DNase I digestion of DNA indicates the stability of DNA. A comparative analysis of DNase I digestion sensitivity of DNA from control and Aβ (1-42) injected rabbit brain was carried out to further understand the stability and to confirm condensation of DNA. Both FC and HDNA from Aβ (1-42) injected aged rabbit brain showed more sensitivity to DNase I digestion compared to their respective controls (Fig 3A-a. 4A and B). This indicates that DNA from both FC and H from Aβ injected rabbits are already damaged hence sensitive to DNase I digestion. But M-DNA from Aβ (1-42) injected aged rabbit brain showed resistant to DNase I digestion compared to control DNA, confirming condensed state of DNA (Fig 3A-a. 4C).

3A-a.3.2. DNA conformation studies

CD indicates the secondary conformation of DNA. The DNA from FC, H and M regions of control rabbit brain showed B-DNA conformation (Fig 3A-a. 5). But DNA from Aβ (1-42) injected rabbit brain regions showed conformational change. (a) FC: DNA isolated from FC showed a decrease in 220 nm positive peak, an increase in 245 nm negative peak and a decrease in 270 nm positive peak indicating C-DNA motif conformation (Fig 3A-a. 5a). C-DNA motif conformation was assigned using references (Ivanov *et al*., 1973; Zimmer *et al*., 1971). (b) H: CD spectra of DNA from Aβ (1-42) injected aged rabbit brain regions H showed an increase in the 220 nm positive peak, a decrease in 245 nm negative peak and an increase in 270 nm positive peak indicating π-DNA conformation (Fig 3A-a. 5b). π-DNA conformation was

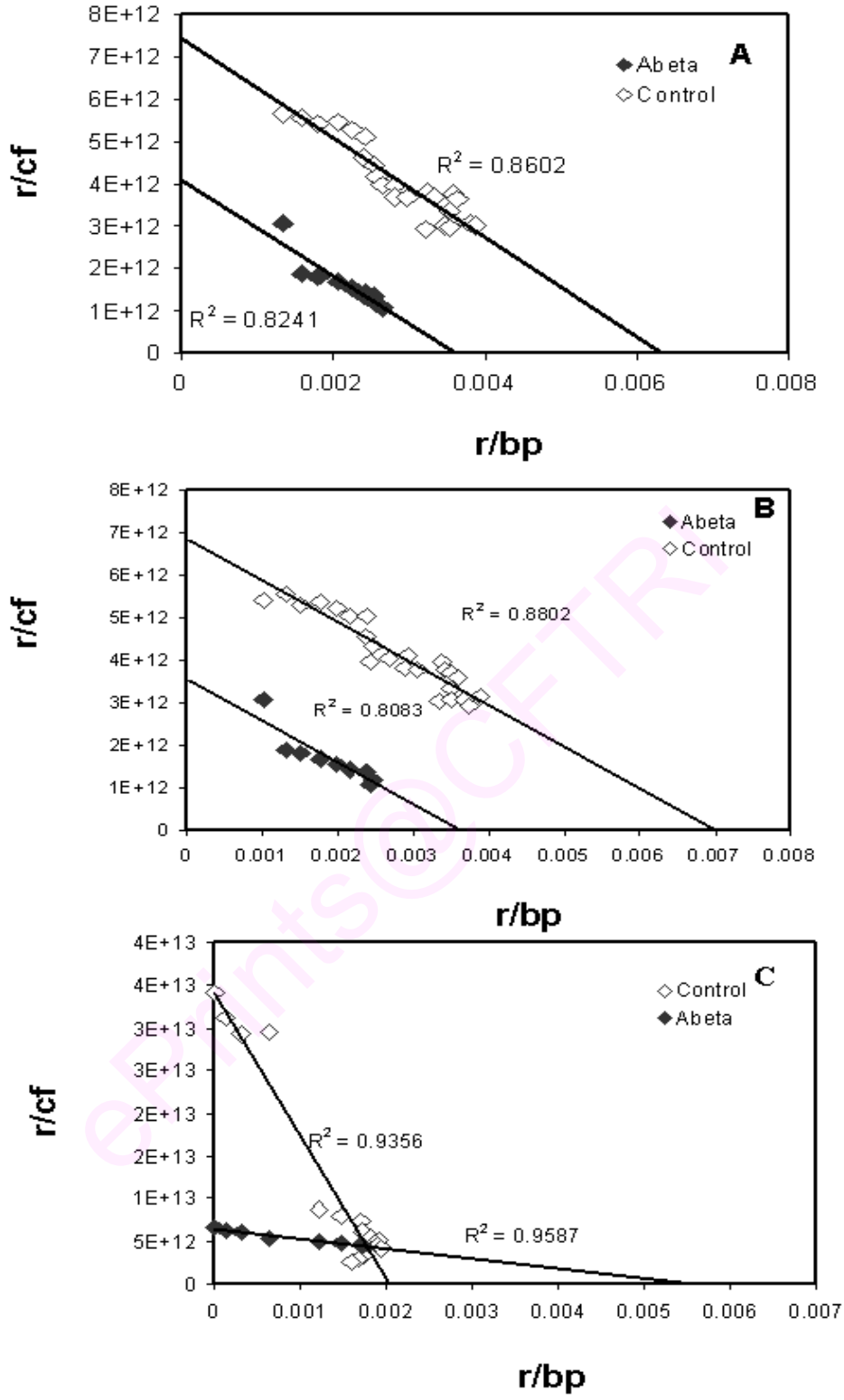


Fig. **3A-a.3.** Scatchard plot showing number of EtBr molecules per base pair of DNA fromfrontal cortex, hippocampus and midbrain regions of Rabbit brain. (A) Frontal cortex DNA; (B) hippocampus DNA, (C) midbrain DNA.

Table **3A-a.**1: The data indicates the number of EtBr molecules binding per base pair of DNA of isolated from brain regions (*p < 0.05, **p<0.01).

Brain regions	**Control**	**Aβ(1-42)**
Frontal cortex	0.0087 ± 0.00039	0.0013 ± 0.00019 **
Hippocampus	0.0057 ± 0.00011	0.0035 ± 0.00013*
Midbrain	0.0058 ± 0.00028	0.0043 ± 0.00027 *

assigned using the references (Robinson and Wang, 1993; Yang *et al*., 1993). (c) M: CD spectra of DNA from Aβ (1-42) injected rabbit brain M showed slight increase in 220 nm peak and cross over at 248 nm negative peak and decrease in 270 nm positive peak indicating Ψ-DNA conformation (Fig 3A-a. 5c). Ψ- DNA conformation was assigned using the reference (Khakade and Rao, 1997). Ψ-DNA indicates condensed state of DNA (Khakade and Rao, 1997). This is the first evidence to show that Aβ induces conformation changes in DNA under *in vivo* condition and has great relevance to understand Aβ role in neurodegeneration through genomic biology.

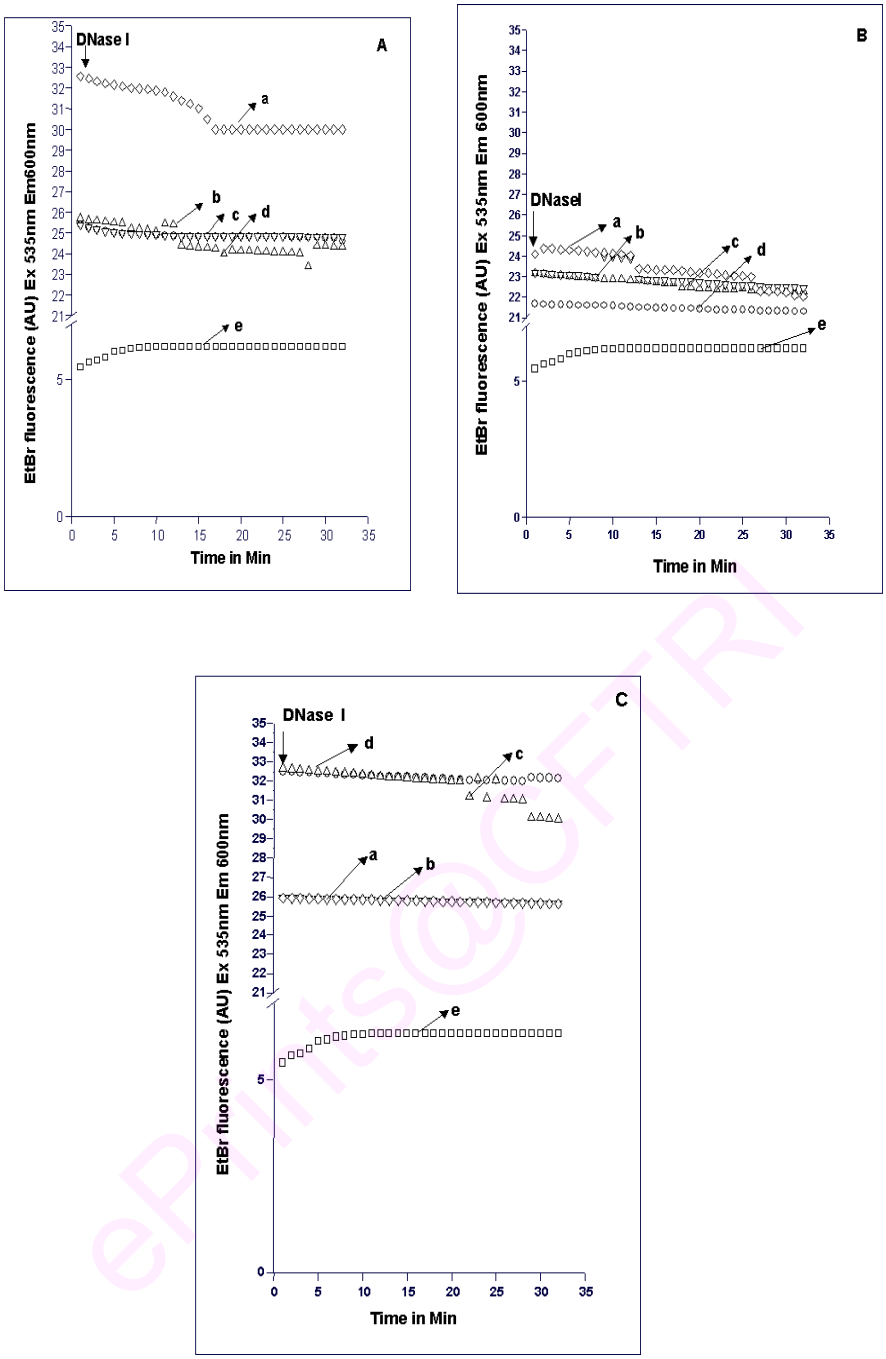


Fig. 3A-a.4. DNase I sensitivity assay to study DNA stability. A DNA from Saturated mixture of Different regions of both control and Aβ (1-42) injected aged rabbit brain was treated with DNase I and fluorescence emission was recorded with time excitation at 535 nm and emission at 600 nm. (A) Frontal cortex DNA; (B) hippocampus DNA; (C) midbrain. (a) Aβ (1-42) injected DNA+ DNase I, (b) Aβ (1-42) injected DNA, (c) control DNA+ DNase I, (d) control DNA, (e)-EtBr alone.

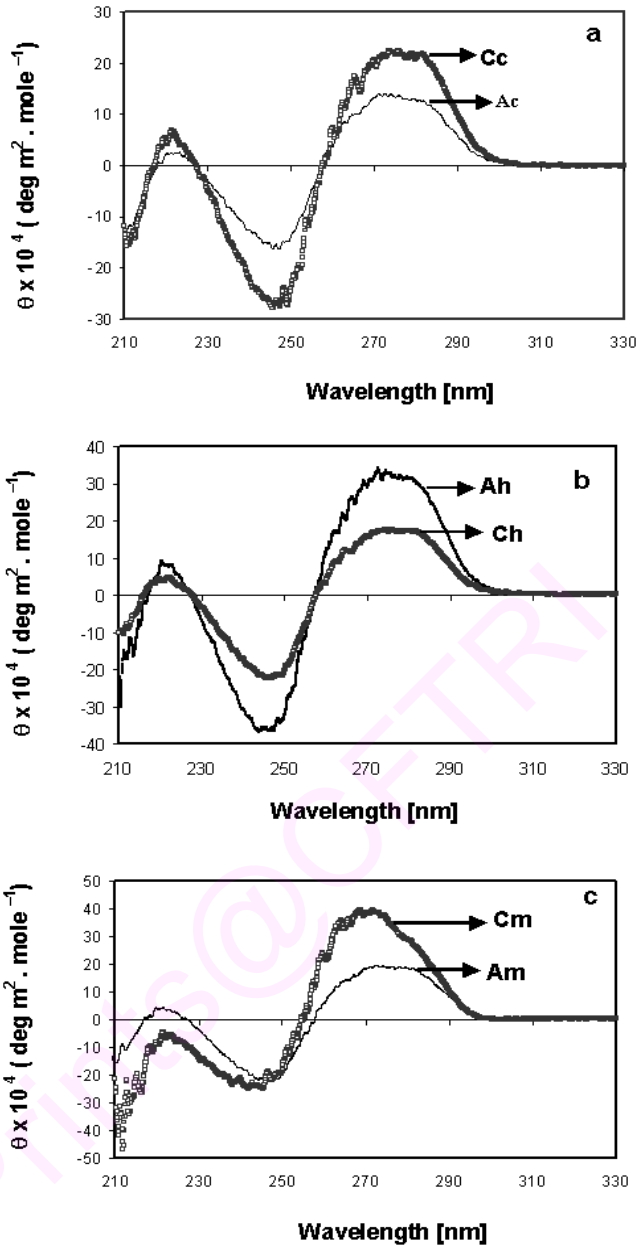


Fig. 3A-a. 5. Circular dichroism (CD) spectra of DNA isolated from frontal cortex, hippocampus and midbrain regions of control and Aβ (1-42) injected rabbit brain. The recordings were performed in 0.01 M Tris–Cl buffer, pH 7.4. Each spectrum represents the average of three recordings. CD was performed DNA from six control and six Aβ (1-42) injected aged rabbit brain and one spectrum from each group is represented. The DNA from control animal showed B-DNA conformation where as C type,π□-DNA and Ψ-DNA conformation was observed in Aβ (1-42) injected FC,H and M regions of aged rabbit brain. (a) Frontal cortex, (b) hippocampus, (c) midbrain. Cc—control cortex DNA, Ac—Aβ (1-42) injected cortex DNA. Ch—control hippocampus. DNA, Ah—Aβ (1-42) injected hippocampus DNA. Cm—control midbrain DNA and Am— Aβ (1-42) injected midbrain DNA.

3A-a.3.3. MRI of aged rabbits

Fig. 3A-a.6 indicates MRI of aged rabbits brain injected with Aβ (1-42) and saline. There are no significant changes in the thickness of frontal lobe, H, M and lateral ventricle between saline control and Aβ (1-42) injected rabbits (Table 3A.2).

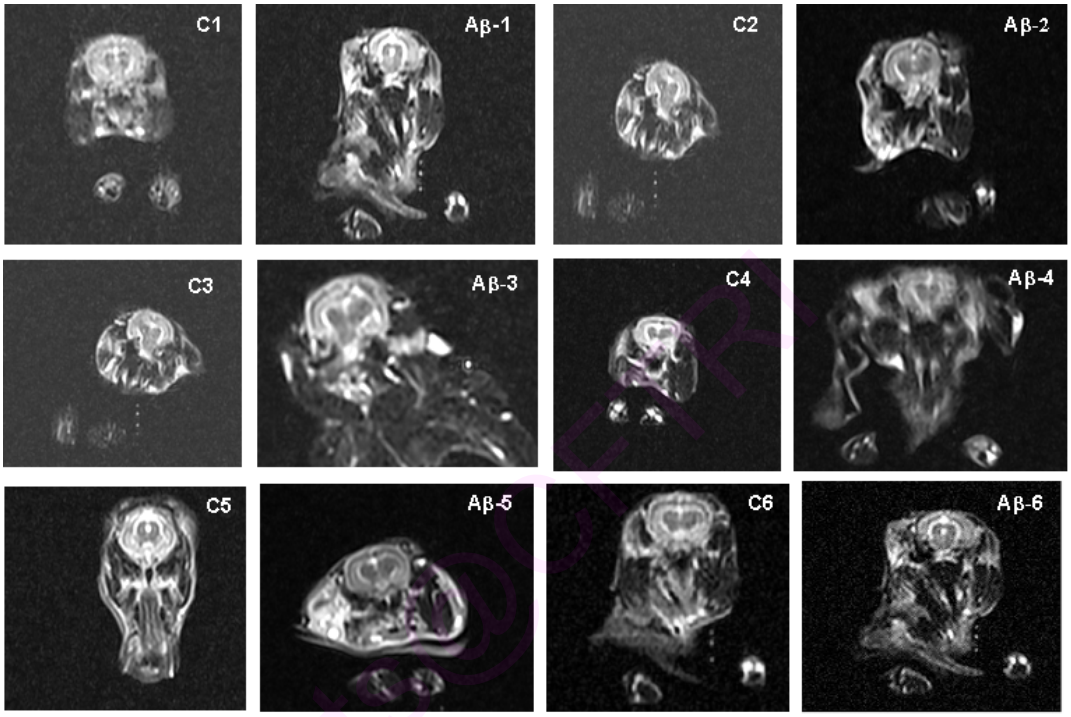


Fig. 3A-a.6. Representative photos of coronal T1 brain slices. C1–C6 are control rabbits. Aβ1–6 shows the rabbits injected with Aβ (1-42). Image analysis was done by comparing the anatomy of control scans with scans obtained after injection of Aβ (1-42). The rabbits injected with Aβ (1-42) did not show any changes in the ventricle size and temporal lobe volume.

3A-a.Table 2: Changes regenerated as average in the thickness (mm) of different brain regions of Aβ(1-42) injected and control rabbits. Legend: C- Control, Aβ- Aβ(1-42). Inference from above table is that there is no significant change in the thickness of frontal lobe, hippocampus and midbrain of Aβ(1-42) injected rabbit brain compare to control.

Brain regions	**C1**	**Aβ1**	**C2**	**Aβ2**	**C3**	**Aβ3**	**C4**	**Aβ4**	**C5**	**Aβ5**	**C6**	**Aβ6**
Frontallobes Left	1.5	1.7	1.5	1.6	1.9	1.6	2.0	1.6	1.6	1.4	1.3	1.2
Right	1.5	1.6	1.7	1.6	1.7	1.7	1.7	1.9	1.7	1.7	1.6	1.6
Hippocampus Left	3.6	3.4	3.1	3.4	3.8	3.6	3.7	3.4	3.4	3.4	3.3	3.2
Right	3.4	3.7	3.2	3.2	3.7	3.7	3.9	3.5	3.5	3.3	3.5	3.4
Midbrain	5.0	5.1	8.1	7.5	6.5	6.4	7.5	7.5	7.4	7.3	7.4	7.3
Lateral ventricle	1.7	1.6	1.6	1.6	1.4	1.8	1.5	1.7	1.7	1.6	1.5	1.6

3A-a.4. Discussion

Loss of neurons is hallmark pathology of neuodegenerative disorders and there is growing evidence suggesting that apoptosis may play role in neuronal cell death (Gupta *et al*., 2006; Su *et al*., 1997). Damage or alteration in DNA appears to be a universal phenomenon both in normal ageing and age related disorders (Rao, 1997). DNA is subjected to damage in cells by both endogenous and exogenous events that result in DNA instability (Rao, 1997). The DNA instability in AD brain may be due to the production of mismatched base pairs, strand breaks, and cross-links between DNA and proteins and DNA helicity changes (Anitha *et al*., 2002). It is hypothesized that Aβ plays a significant role in genomic instability (Suram *et al*., 2006; Hegde *et al*., 2004; Suram *et al*., 2007). There are limited studies on integrity of DNA topology in Neurodegenerative brain (Anitha *et al*., 2002; Hegde *et al*., 2006). The DNA instability and repair system in neurodegeneration is still not clearly understood. The fundamental study from our lab showed, that Aβ localizes in nuclear region in the apoptotic neurons only in the hippocampus of AD brain (Hegde *et al*., 2004). The major question still unanswered in the above paper is, why does Aβ translocates to nucleus of apoptotic neurons only? Also, it is still not clear whether Aβ induces apoptosis in neurons or apoptotic neurons favor the translocation of cytoplasmic Aβ to nuclear region? (Hegde *et al*., 2004). Hegde *et al*. (2004) clearly showed that Aβ (1-42) binds to and destabilizes DNA. It was also reported that Aβ (1-42) converted B-DNA to Ψ-DNA conformation. And Ψ- conformation mimics Z-DNA conformation. The presence of Z-DNA was observed in hippocampus of AD brain (Anitha *et al*., 2002). Further, recent studies provided new evidence that Aβ□behaves like a endonuclease and causes nicks in DNA leading to DNA instability (Suram *et al*., 2007). In our present study, DNA stability parameters like Tm, EtBr and DNase I digestion sensitivity of DNA from FC and H showed that Ab injection causes DNA damage in aged rabbit brain. But M DNA is in condensed state, as evidenced by an increase in Tm and decrease in EtBr binding and DNase I resistant. Thus, it is clear from our study that Aβ (1-42) causes DNA damage in FC and H, where as Aβ (1-42) induces condensation of in M region in rabbit brain. Further, Ghribi *et al*. (2001) showed that intracisternal injection of Aβ (1-42) to aged rabbit elevated gadd 153 expression, which plays an important role in DNA damage. Ohyagi *et al*. (2005)

reported that intracellular Aβ (1-42) activates p53, resulting in p53-dependent apoptosis in cells. Raff (1992) showed that neuronal cells treated with Ab exhibit morphological and biochemical characteristics of apoptosis, including membrane blebbing, compaction of chromatin and interchromosomal DNA fragmentation. Lakshman *et al*. (2005) reported new data on translocation of Aβ from cytosol to nucleus through Aβ□related Death-Inducing Protein (AB-DIP). The activation of AB-DIP is proposed to be linked Caspase-9 and this has significant role in cell death through apoptosis pathway, but the mechanism is still not clear. The proposed neurotoxicity mechanisms for Aβ are diverse and there are still no unifying mechanisms for neurotoxicity and genotoxicity. The DNA stability changes in neurodegeneration are still a challenging pathway to be explored. The main concept is to understand, whether Aβ (1-42) causes any conformational change in DNA? and to map its relevance to cell dysfunction. Our results on DNA conformation showed that Aβ induces C-type DNA and π-DNA in FC and H regions respectively. The M-DNA is in ψ-DNA conformation, which will be in left-handed helix, resembling Z-DNA in conformation features (Thomas and Thomas, 1989). Further, Thomas and Thomas (1989) reported that c-DNA is an ordered, twisted and tight packing arrangement of double helix and immunologically close to Z-DNA family. ψ-DNA is also in left handed in conformation like Z-like DNA. This indicates that Ab induces DNA topological changes under *in vivo*. These changes are similar to the DNA conformational changes observed in AD brain hippocampus (Suram *et al*., 2002). To the best of our knowledge, this is a fundamental contribution in understanding the role of Aβ (1-42) in genomic biology of rabbit brain, which closely mimics the AD brain. It is curious to know, whether Ab induces cellular atrophy as it is causing cell death events. Price and Calderwood (1992) reported that the levels of Aβ (40)/Aβ(1-42) in AD brain correlates with the frontal lobe atrophy as revealed by MRI. Also studies have shown a positive correlation between the Aβ load and the reduction in brain volume and atrophy in AD patients (Archer *et al*., 2006; Silbert *et al*., 2003). Fox *et al*. (2005) showed that immunization of AD patients with anti-Ab (1-42)(AN1792/QS-21) antibody reduced the loss of volume in cerebral region, indicating a relation between Aβ load to neuronal loss. Nakamura *et al*. (2001) reported that intracerebroventricular infusion of Aβ (1-42) in rats resulted in

progressive reduction in brain functions including behavioral, histological and neurochemical changes. In our study, we observed no change in the thickness of temporal lobe, hippocampus and dilation of the ventricle volume in Aβ (1-42) injected rabbit brain for 25 days. Our findings are partially substantiated Josephs *et al*. (2008). They reported that Ab burden is not associated with brain atrophy in AD patients and they concluded that rate of brain volume loss is not determined by the amount of insoluble Aβ in the gray matter. Further, Stein-Behrens *et al*. (1992) showed that the rats injected with Aβ (25–35) did not show any damage in brain structure. Also the rhesus monkeys stereotypically injected with Aβ (40) did not show any cellular changes resembling AD pathology (Podlisny *et al*., 1992). Since the process of neurodegeneration itself is a slow process and may take a decade or even more to pronounce the gross changes in the brain structure. We assume that we did not see any significant MRI changes, since ours is only 25 days after Aβ (1-42) injection, which is short period to show gross structural changes as evidenced by MRI. However, our very recent studies insighted the brain structural changes are seen after Aβ(1-42) injection with time scale of 45 days duration studied by 1.5 T MRI (unpublished data). Based on our novel findings and reports from literature we propose, a new hypothesis on the mechanisms of Aβ (1-42) induced genomic instability and its relevance to neuronal dysfunction (Fig. 7). Aβ presents initially in the cytoplasm and was reported to be in the cell debris after cell death finally. Recently, it has been shown that Aβ translocates from cytoplasm to nuclear region in AD brain. The evidences have shown that Aβ may induce DNA instability either through oxidative stress mediation or through directly binding to DNA. Further, metals like Al, Cu and Fe accumulate in the brain in complex with Aβ may favor the DNA damage. The damaged DNA will have lower activation energy and may prone for conformational change upon Aβ binding? In the present study, we have evidenced the changes in DNA conformation from B-DNA to C-type, π-DNA and Ψ-DNA form in FC, H and M respectively in Aβ (1-42) injected aged rabbit brain. We presume that metals accumulation and Aβ load, together play a significant role in altering the genomic biology of brain. We hypothesize that change in the DNA conformation may alter the transcription factors binding leading to cell dysfunction. All these changes may lead to altered gene expression favoring neuronal dysfunction.

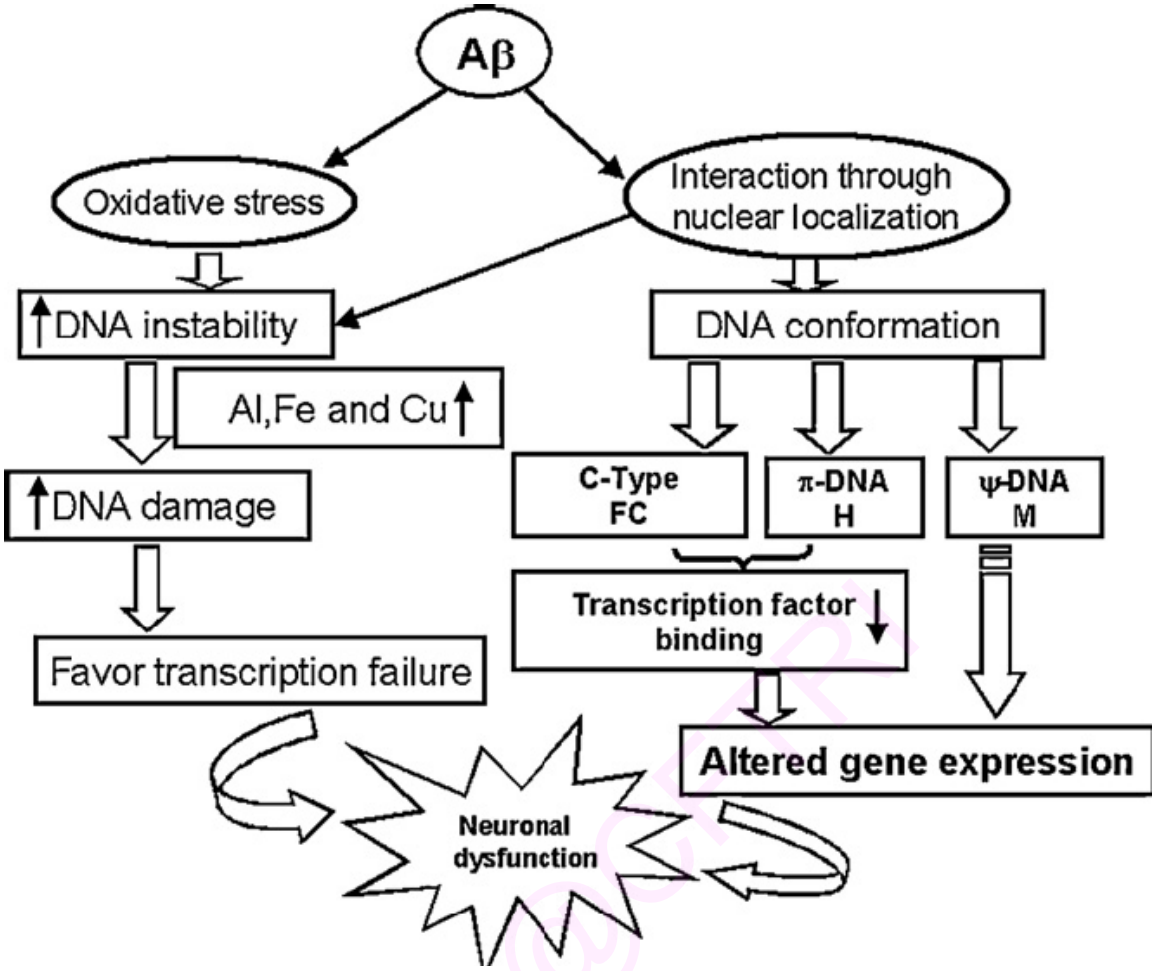


Fig. 3A-a. 7. Our proposed hypothesis on mechanism of Aβ (1-42) induced genomic instability and its relevance to neuronal dysfunction is presented below. Aβ normally present initially in the cytoplasm and reported to be in the cell debris after cell death finally. Recently, it has been shown that Aβ translocates from cytoplasm to nuclear region in AD brain. The evidences have shown that Aβ may induce DNA instability either through oxidative stress mediation or through direct binding to DNA. Further, metals like Al, Cu and Fe accumulate in the brain in complex with Aβ and may favor the DNA damage. The damaged DNA will have lower activation energy and may prone for conformational change upon Aβ binding? In the present study, we have evidenced the changes in DNA conformation from B-DNA to C-type, π-DNA and Ψ-DNA form in FC, H and M respectively in Aβ (1-42) injected aged rabbit brain. We presume that metals accumulation and Aβ load, together play a significant role in altering the genomic biology of brain. We hypothesize that change in the DNA conformation may alter the transcription factors binding leading to cell dysfunction. All these changes may lead to altered gene expression favoring neuronal dysfunction.

Chapter 3A-b

Studies to understand the preventive role of indigenous plant extracts in Aβ(1-42) induced DNA damage

Chapter 3A-b Studies to understand the preventive role of Indigenous plant extracts against Aβ(1-42) induced DNA damage

3A-b-1.Introduction

Curcumin is a polyphenolic compound found in rhizome of turmeric (Curcumin longa). The curcumin is used as spice and as a coloring agent in Indian food. Curcumin has a long history for its use in Indian traditional medicine; ayurveda [Goel *et al*., 2008; Aggarwal *et al*., 2007]. The consumption of curcumin has been attributed to the lower incidence of gastro-intestinal mucosal cancers [Mohandas and Desai, 1999; Sinha *et al*., 2003]. Curcumin is also shown to have a potential to delay the progression of AD (Ringman *et al*., 2005; Wakade *et al*., 2009; Jung *et al*., 2006). Curcumin is reported to inhibit the Aβ oligomerization and fibril formation [Ono *et al* ., 2004; Yang *et al*., 2005]. The studies have shown that curcumin inhibits neuronal damage [Shukla *et al*., 2003], reduce the oxidative damage (Lim *et al*., 2001) and prevents *in vivo* amyloid accumulation in AD transgenic mice [Yang *et al*., 2005]. Garcia-Alloza *et al*. (2007) showed that curcumin crosses the blood brain barrier (BBB) as evidenced in APPSwe/PS1dE9 transgenic mice studies. The naturally occurring dyes such as Congo red (CR), chrysamine G (CG) and curcumin are known to bind Aβ with greater affinity (Lorenzo and Yankner 1994, Yang *et al*., 2005). But interestingly, curcumin is known to inhibit the fibril formation and lower the neurotoxicity induced by Aβ fibrils (Yang *et al*., 2005, Ono *et al*., 2004). The backbone in the curcumin between two aromatic groups. Curcumin contains 3-methoxy 4-hydroxy substitutions on its aromatic groups. This aromatic substitution is capable of taking part in hydrogen bonding and helps in binding to Aβ and inhibiting the Aβ aggregation. But the bioavailability of orally administered curcumin is less due to its hydrophobic nature and hence the therapeutic effects are limited (Sharma *et al*., 2001). Its less bioavailability is due to its total insolubility in water. Since the oral administration of curcumin, found low levels of parent compound and its glucouronide and sulphate conjugates in peripheral and portal circulation are reported (Garcea *et al*., 2004). To improve the bioavailabilty of curcumin, nano-curcumin concept approach has been proposed. The nano-curcumin has the property of being dispersible in aqueous media, which helps in solving the dissolution problem in

aqueous media. This property provides an opportunity for nanoparticle based drug delivery. The nano curcumin finds its use in systemic therapy of human cancers and neurodegenerative disorders such as AD (Lim *et al.,* 2001; Park *et al.,* 2002; Ono *et al* ., 2004; Yang *et al.,* 2005) and cystic fibrosis [Egan *et al*., 2004; Lipecka *et al*., 2006]. Vanillin is a flavoring molecule used in food industry. The health benefits on Vanillin and its derivative VG is not studied. The biology of *C.asiatica* in brain disorders is not clear. The recent reports that showed that the extract of *C.asiatica* protects against gamma-radiation induced DNA damage [Joy and Nair, 2009]. There are reports that oxidative stress has a significant role in neurodegenerative disorders and antioxidants have shown promise in preventing the onset of neurodegenerative disorders [Bouhlel *et al*., 2009; Vostálová *et al*., 2010 ; Stagos *et al*., 2005]. In this present study, we report the effect of curcumin, curcumin derivatives, *C.crista* and *C.asiatica* on Aβ(1-42) DNA nicking property. Also, report on the of effect of CG and VG on secondary conformation of Aβ (1-11, 1-28 and 25-35) fragments.

3A-b.2.Materials and methods

Supercoiled plasmid DNA (pUC18) (Cesium chloride purified) and DNA molecular markers were purchased from Bangalore Genei, India. Aβ(1-11), Aβ(1-28), Aβ(25-35), and Aβ(1-42) were purchased from Sigma, USA. TRIS (hydroxymethyl) and agarose from SRL India. Glacial acetic acid, hydrochloric acid, EDTA, were purchased from BDH chemicals, India. Ethidium bromide was obtained from ICN. Curcumin, CG and VG were courtesy of Dr. P.Srinivas, CFTRI. *C.crista* was brought from Western ghats. *C.asiatica* was purchased from the local market, Mysore. Both *C.crista* and *C.asiatica* were identified by authenticated botanist. The details on preparation of the aqueous extract these two plants were presented in chapter 4B and chapter 4C.

3A-b.2.1 Effect of amino acids derivatives of curcumin on DNA nicking activity of Aβ(1-42)

ScDNA (0.5µg) was incubated with 50µM of different fragments of Aβ(1-42) in presence of both amino acids derivatives of curcumin (Cur-Ala, Cur-Leu, Cur-Ile, Cur-Lys Cur-Phe and Cur-Val,) and 1mM $MgCl_2$ for 12 h in Tris-Cl buffer at pH 7.4 at 37^0C. The incubated samples were electrophoressed on 1% agarose using TAE

(Tris –acetic acid -EDTA) (pH8.4). The gel was stained with 1µg/ml of ethidium bromide in cold water for one h and de-stained in using triple distilled water and documented using gel documentor and densitometry was recorded using the inbuilt software system.

3A-b.2.2. Effect of *Caesalpinia crista* and *Centella asiatica* aqueous leaf extracts on DNA nicking activity of Aβ(1-42)

ScDNA (0.5µg) was incubated with 50µM of Aβ(1-42), 25, 50, and 100µ□g of *Caesalpinia crista* and *Centella asiatica* aqueous leaf extracts in10mM Tris-Cl pH 7.4 at 37 ^{0}C for 12 hours. Incubated samples are electrophoressed on 1% agarose using TAE (Tris –acetic acid -EDTA) (pH8.4). The gel was stained with 1µg/ml of ethidium bromide in cold water for one hour and de-stained in using triple distilled water and documented using gel documentor and densitometry was recorded using the inbuilt software system.

3A-b.2.3. Circular dichroism spectroscopy to study effect of CG and VG on the secondary conformation of Aβ (1-11), Aβ (1-28) and Aβ (25-35)

50µM of Aβ (1-11), Aβ (1-28) and Aβ (25-35) were incubated with CG and VG in the following ratios, 1: 0.1, 1:1, 1:2 and 1:5 for 12 h at 37 ^{O}C in 10mM Tris-Cl, pH7.4. The secondary conformation of Aβ (1-11) and Aβ (1-28) was recorded on JASCO J 700 Spectropolarimeter at 25 ^{O}C, with 2mm cell length with wavelength scan between 195 and 260nm.

3A-b.3.Results

3A-b.3.1. Studies on the effect of curcumin and amino acids derivatives of curcumin on Aβ(1-42) DNA nicking activity

The results showed that curcumin, amino acids derivatives of curcumin Cur-Ala, Cur-Ileu, Cur-Leu, Cur-Phe, Cur-Lys, Cur-Val did not prevent the Aβ(1-42) DNA nicking activity (Fig 3A-b.3.1).

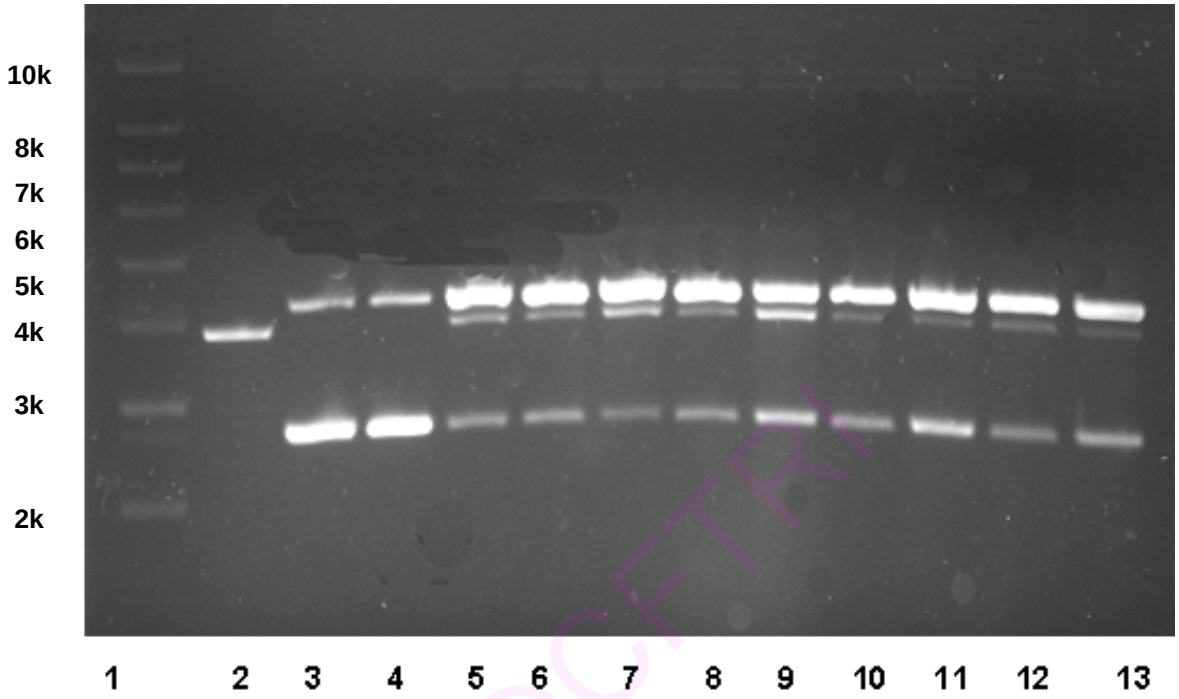


Fig 3A-b.3.1: Effect of curcumin derivatives on DNA nicking property of Aβ(1-42) : 1-1kb marker, 2- Linearized DNA-EcoRI digested, 3- scDNA, 4-scDNA+ DMSO, 5- scDNA+ Aβ (1-42)+ Mg^{2+}+ DMSO, 6- scDNA+ Aβ (1-42)+ Mg^{2+}, 7- scDNA+ Aβ (1-42)+ Mg^{2+}+ Curcumin, 8- scDNA+ Aβ (1-42)+ Mg^{2+}+ Cur-Ala, 9- scDNA+ Aβ (1-42)+ Mg^{2+}+ Cur-Ile, 10- scDNA+ Aβ (1-42)+ Mg^{2+}+ Cur-Leu, 11- scDNA+ Aβ (1-42)+ Mg^{2+}+ Cur-Lys, 12- scDNA+ Aβ (1-42)+ Mg^{2+}+ Cur-Phe,13- scDNA+ Aβ (1-42)+ Mg^{2+}+ Cur-Val.

3A-b.3.2. Studies on the effect on aqueous leaf extracts of *C.crista* and *C.asiatica* on Aβ(1-42) DNA nicking activity

The agarose gel was used to demonstrate whether the aqueous leaf extracts of *C.crista* and *C.asiatica* can inhibit Aβ(1-42) DNA nicking activity (Fig 3A.3.2-B). The results showed that 5µg *C. crista* inhibited nicking activity of Aβ(1-42), whereas higher concentrations of 25 and 50 µg *C. crista* enhanced DNA nicking activity of Aβ(1-42)(Fig 3A.b.3.2). However, the 5, 25 and 50 µg *C.asiatica* aqueous

leaf extract did not inhibit the DNA nicking activity of Aβ(1-42)(Fig 3A-b.3.2-B). The nuclease inhibitor ATA inhibited the DNA nicking activity of Aβ(1-42).

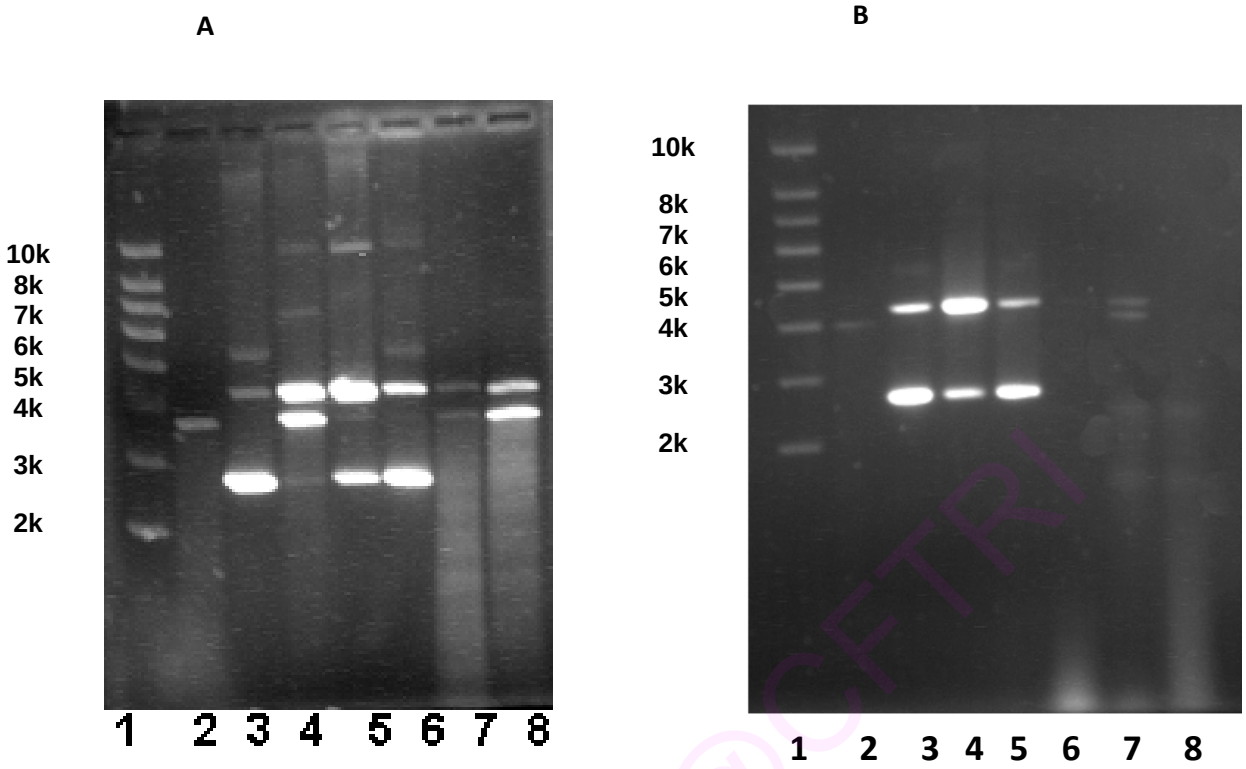
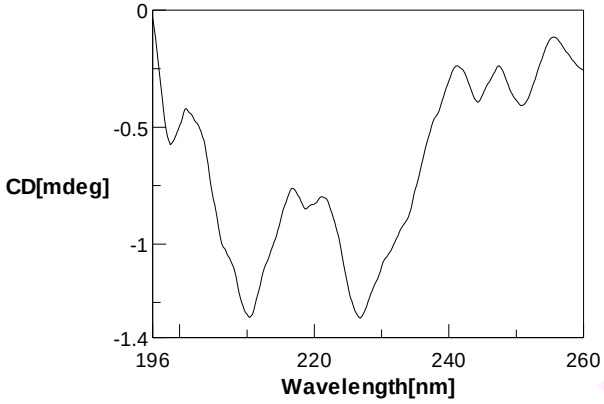


Fig 3A-b.3.2: Effect of aqueous leaf extracts of *C.crista* and *C.asiatica* on DNA nicking property of Aβ(1-42): A:1- 1kb marker, 2- Linearized DNA-EcoRI digested, 3- scDNA, 4- scDNA+ ATA, 5- scDNA+ Aβ(1-42), 6- scDNA+ Aβ(1-42)+5µg *C.crista*, 7- scDNA+ Aβ(1-42)+25µg *C.crista,* 8- scDNA+ Aβ(1-42)+50µg *C.crista.* B:1- 1kb marker, 2- Linearized DNA-EcoRI digested, 3- scDNA, 4- scDNA+ Aβ(1-42), 5-- scDNA+ Aβ(1-42)+ ATA, 6- scDNA+ Aβ(1-42)+5µg *C.asiatica*, 7- scDNA+ Aβ(1-42)+25µg *C.asiatica,* 8- scDNA+ Aβ(1-42)+50µg *C.asiatica*.

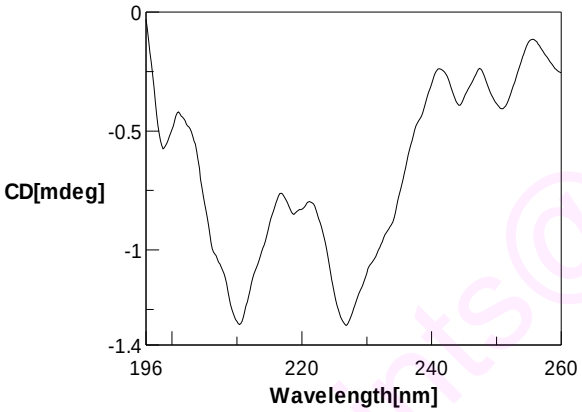
3A-b.3.3. Effect of CG and VG on secondary conformation of Aβ(1-11), Aβ(1-28) and Aβ(25-35)

The conformation of Aβ is important to understand the toxic effects. Recently accepted hypothesis on Aβ in soluble oligomers form are more toxic than insoluble protofibrils and plaques. We have interacted the Aβ(1-11), Aβ(1-28) and Aβ(25-35) with CG and VG at different ratios and recorded the secondary conformation of Aβ(1-11), Aβ(1-28) and Aβ(25-35) and expressed in percentage of Alpha helix, Beta sheet and Random coil (Fig 3A-b.3, Table 3A-b.1). Both VG and CG had changed the

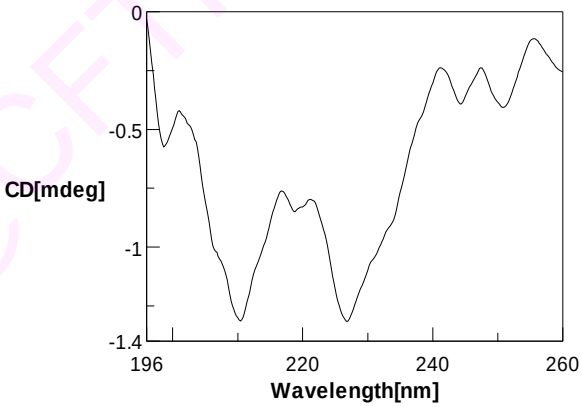
random coil of Aβ(1-28) to beta sheet (Table 3A-b-1). In general, there is an increase in beta sheet conformation at Aβ(1-28): at CG, 1:0.1 and Aβ(1-28): at VG, 1:0.1 ratios and there is no significant increase in beta sheet conformation at 1:1,1:2 and 1: 5 ratios of both CG and VG. But the conformation of Aβ(1-11) upon binding to CG increased with the Alpha helix (39%) conformation at Aβ(1-11): CG, 1:0.1, at Aβ(1-11): CG, 1:1- 32%, at Aβ(1-11): CG, 1:2 -28% rations. Where as at Aβ(1-11): CG, 1:5 -12% and % beta sheet increased to 40 from 24%. As the % beta sheet conformation increases, there is corresponding decrease in the % random coil and not much change in the % alpha helix conformation. The Alpha helix conformation of Aβ(1-11) upon VG binding at Aβ(1-11): VG, 1:0.1and Aβ(1-11): VG, 1:1 is 5% and 7% respectively where as the at same concentration % beta sheet conformation is 47 and 45 respectively. Interestingly, Alpha helix conformation of Aβ(1-11) upon VG at Aβ(1-11): VG, 1:2 and Aβ(1-11): VG, 1:5 is 30% and 39% respectively and beta sheet conformation at same ratio is 1% and 12% respectively. In case of Aβ(25-35), as the concentration of CG increases there is a slight increase in beta-sheet and corresponding decrease in the random coil conformation and no much alteration in the alpha-helix. While VG has not altered secondary conformation of Aβ(25-35) significantly compared to control. In above-mentioned results, as the %alpha helix and % beta sheet increases there is corresponding decrease in the % random coil. This data on secondary conformation of amyloid beta conformation suggest that both CG and VG act differently in altering the secondary conformation of amyloid beta (1-11, 1-28 and 25-35) fragments.



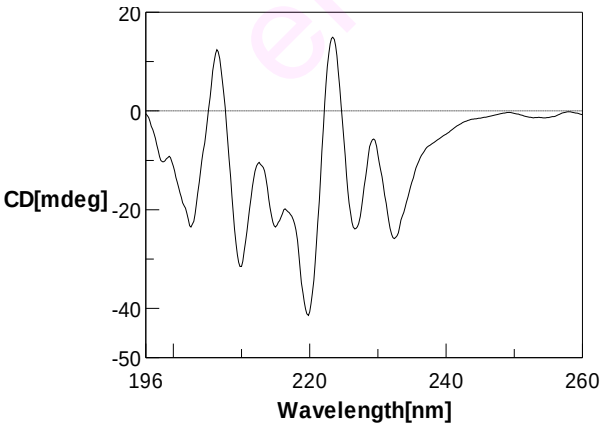
Aβ(1-11)



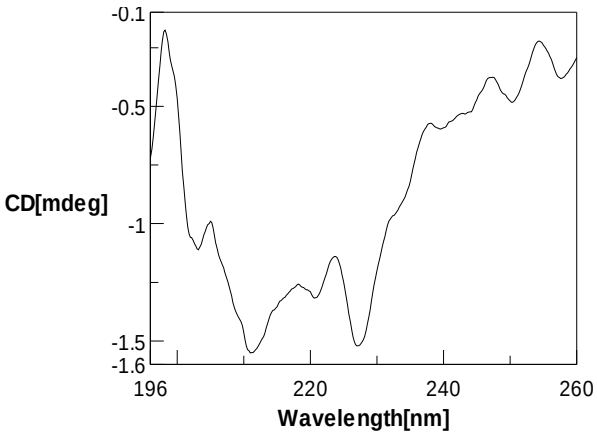
Aβ (1-11) with CG (1:0.1)



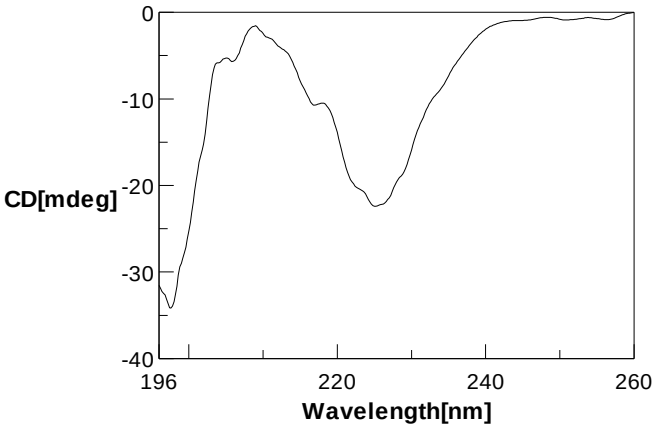
Aβ (1-11) with VG (1:0.1)



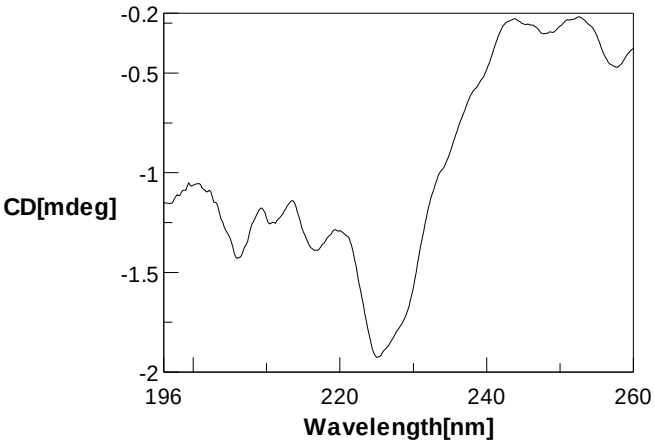
Aβ (1-11) with



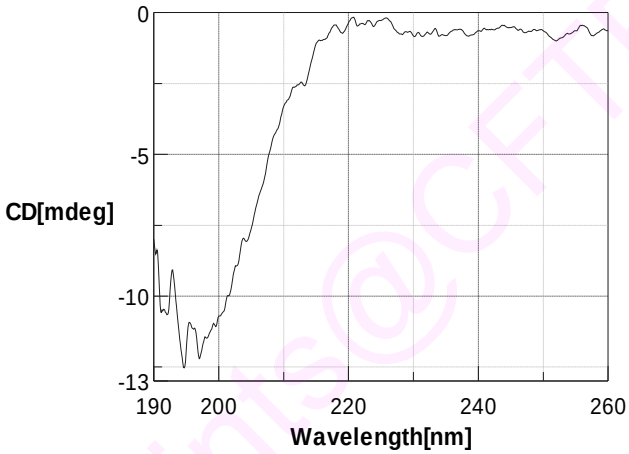
Aβ (1-11) with VG(1:2)



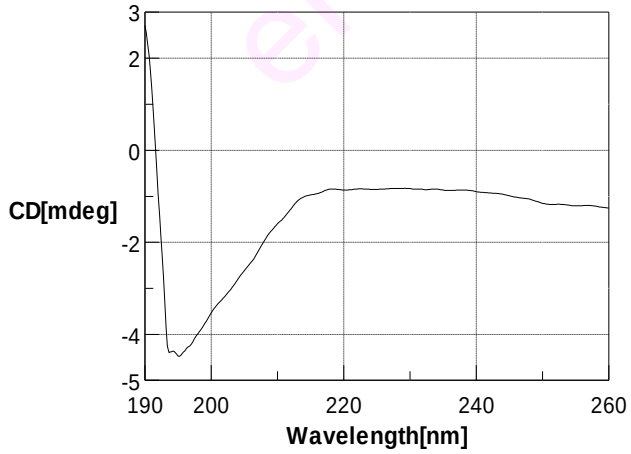
Aβ (1-11) with CG(1:5)



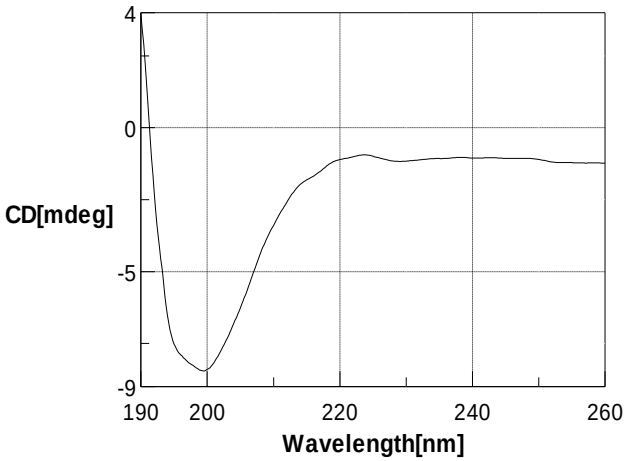
Aβ (1-11) with VG(1:5)



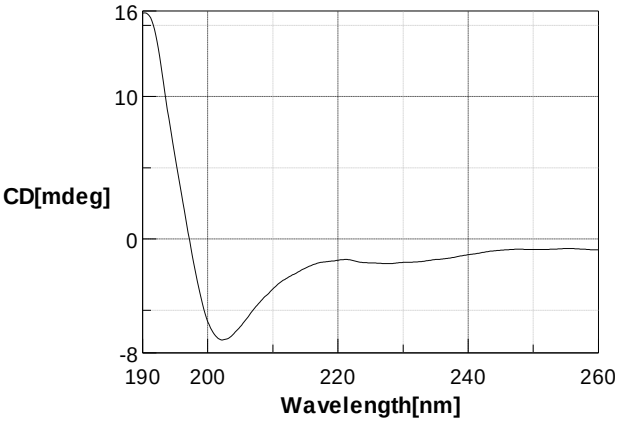
Aβ (1-28)



Aβ (1-28) with CG (1:0.1)



Aβ (1-28) with VG (1:0.1)



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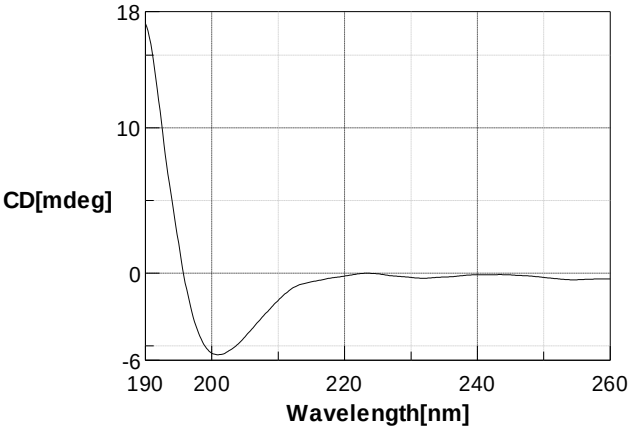
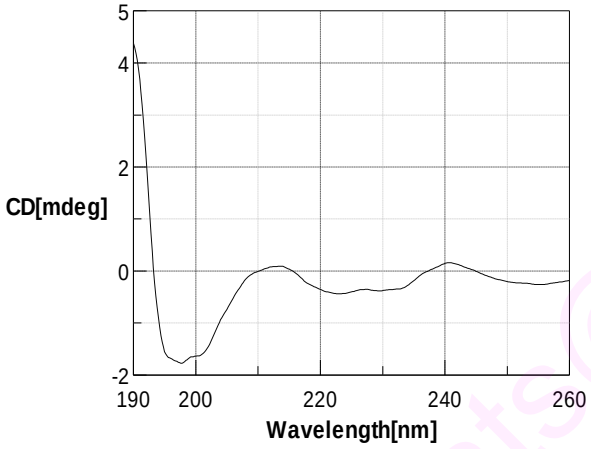
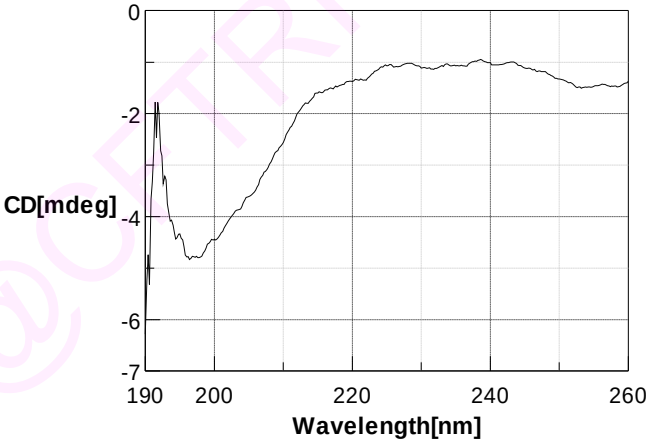


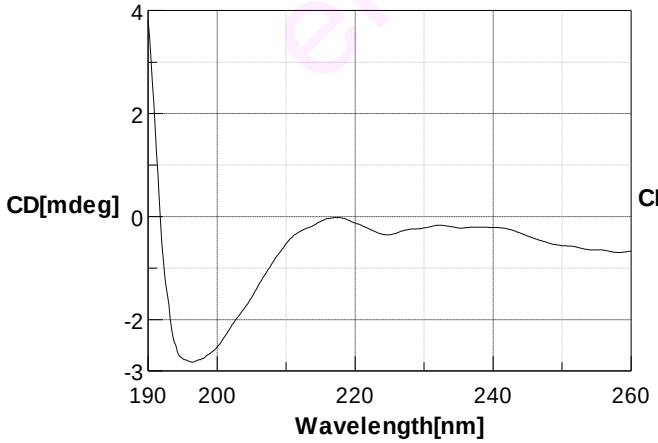
Aβ(1-28)withVG (1:1)



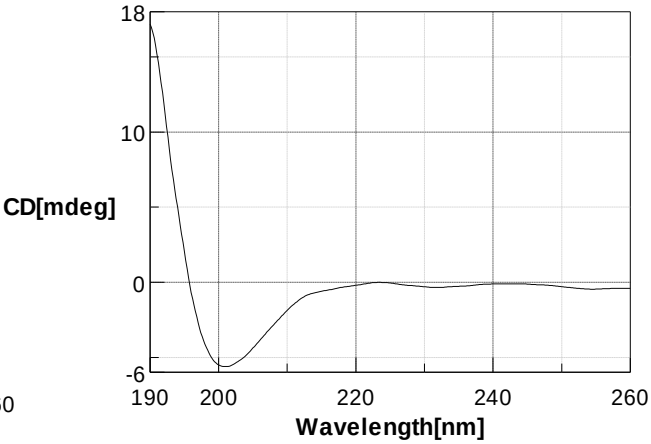
Aβ(1-28) with CG (1:2)



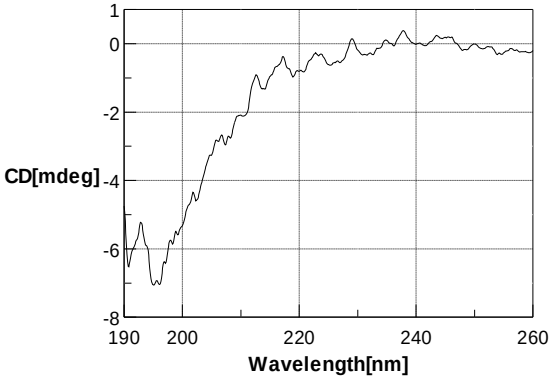
Aβ(1-28) with VG (1:2)



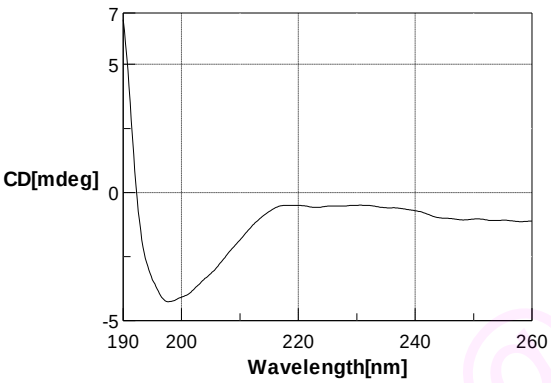
Aβ (1-28) with CG(1:5)



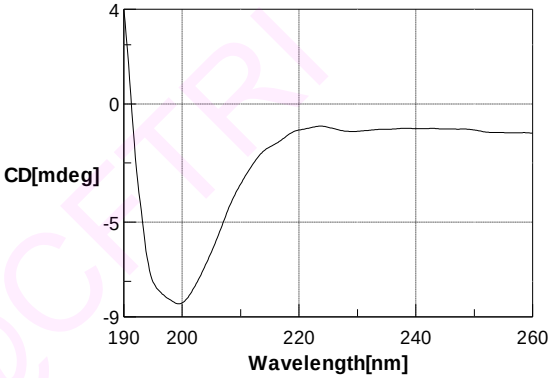
Aβ (1-28) with VG(1:5)



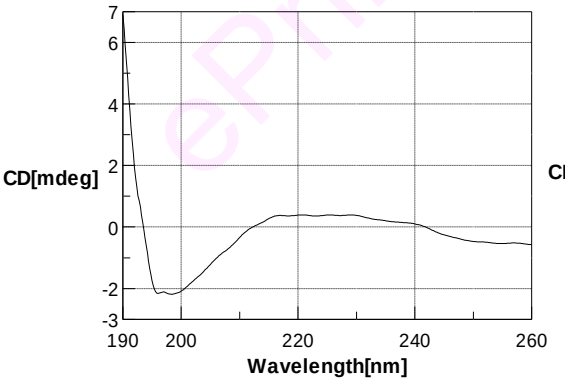
Aβ (25-35)



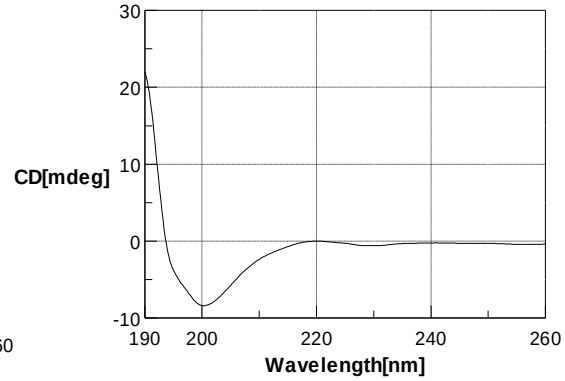
Aβ (25-35) with CG(1:0.1)



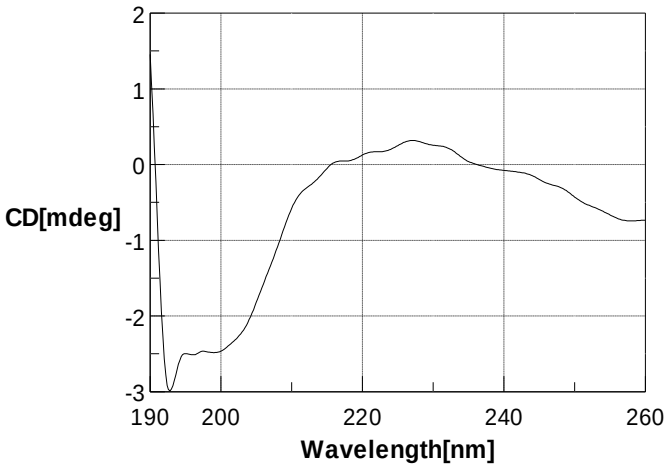
Aβ (25-35) with VG(1:0.1)



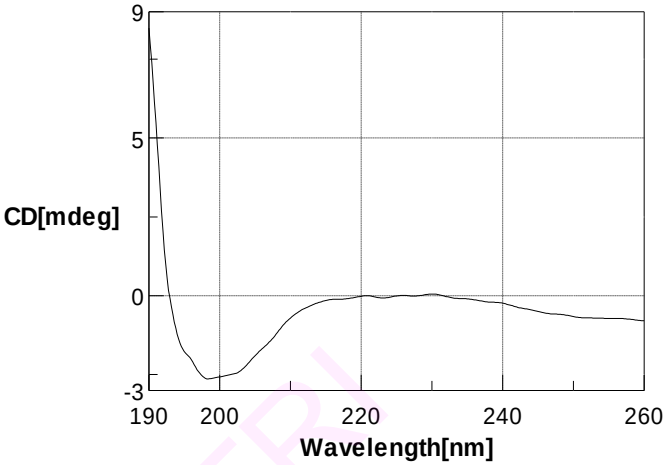
Aβ(25-35) with CG



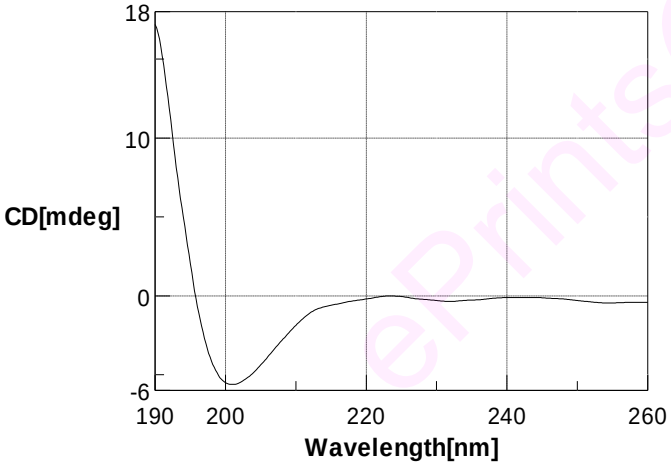
Aβ (25-35) with VG



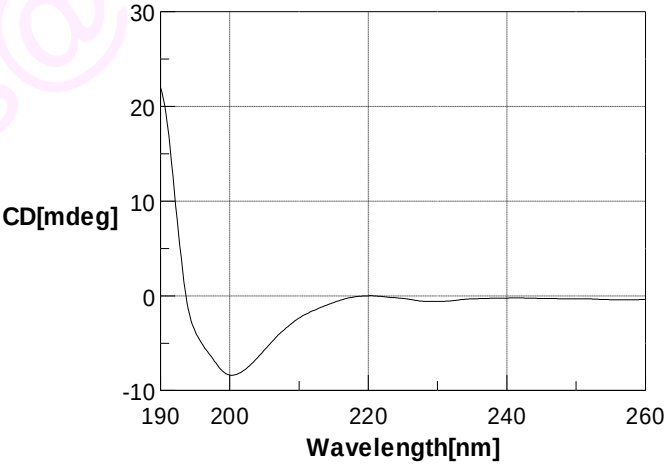
Aβ (25-35) with CG (1:2)



Aβ(25-35) with VG (1:2)



Aβ(25-35) with CG (1:5)



Aβ(25-35) with VG (1:5)

Fig 3A-b.3.3: Efect of CG and VG on the secondary conformation of Aβ (1-11, 1-28 and 25-35) fragments: The secondary conformation was studied by using circular dichroism spectroscopy. The results showed differential effect of CG and VG on secondary conformation of Aβ fragments.

Table 3A-b.1: Effects of CG and VG on the secondary conformation of Aβ(1-11, 1-28 and 25-35) fragments

Sl.No.	Aβ fragments CG/VG	% Alpha helix	% Beta sheet	% Random coil
1	Aβ1-11	5	24	71
2	Aβ1-11:CG,1:0.1	39	10	60
3	Aβ1-11:CG,1:1	32	14	54
4	Aβ1-11:CG,1:2	28	14	58
5	Aβ1-11:CG,1:5	12	40	48
6	Aβ1-11:VG,1:0.1	5	47	48
7	Aβ1-11:VG,1:1	7	45	48
8	Aβ1-11:VG,1:2	39	1	60
9	Aβ1-11:VG,1:5	30	12	58
10	Aβ1-28			
11	Aβ1-28:CG,1:0.1	9	47	44
12	Aβ1-28:CG,1:1	8	48	44
13	Aβ1-28:CG,1:2	8	48	44
14	Aβ1-28:CG,1:5	9	47	44
15	Aβ1-28:VG,1:0.1	7	51	42
16	Aβ1-28:VG,1:1	8	50	42
17	Aβ1-28:VG,1:2	7	50	43
18	Aβ1-28:VG,1:5	8	46	46
19	Aβ25-35			
20	Aβ25-35:CG,1:0.1	4	48	48
21	Aβ25-35:CG,1:1	7	51	42
22	Aβ25-35:CG,1:2	8	50	42
23	Aβ25-35:CG,1:5	7	50	43

24	Aβ25-35:VG,1:0.1	8	48	44
25	Aβ25-35:VG,1:1	8	47	44
26	Aβ25-35:VG,1:2	9	48	43
27	Aβ25-35:VG,1:5	5	47	48

3A-b.4.Discussion

The brain cells which are non-replicating are particularly vulnerable to DNA damage and DNA damage accumulation leads to deleterious effect on neuronal cells (Bohr *et al*., 2007). Recent studies on AD, PD, HD, ALS, XP suggests that oxidative stress and DNA damage are common mechanisms in the neuronal cell death (Kraemer *et al*., 2007; Trushina and McMurray, 2007). The instability of the genome in all the organisms can be induced by exogenous and endogenous agents that induces DNA lesions, DNA breaks etc. (Bohr *et al.,* 2007).The exposure to reactive oxygen species (ROS) may be a risk factor for neurodegenerative disorders and aging (Akbari *et al*., 2006). So, ROS are important genotoxic molecules that are responsible for DNA damage. ROS are known to cause DNA lesions which includes DNA single and double strand breaks, oxidized DNA bases and repaired by base excision repair (BER) (Seeberg *et al*., 1995). Suram *et al*. [2006], in their review suggested that Aβ also acts as a genotoxic molecule involved in DNA damage and neuronal cell death. We have evidenced the DNA nicking activity of different amyloid beta fragments [see chapter 2]. The data on DNA nicking activity of different amyloid beta fragments suggests that amyloid beta fragments directly damage the DNA. Since, curcumin is reported to have neuroprotective effects, it made us curious to understand the curcumin and its derivatives effects on DNA nicking activity of amyloid beta fragments. The curcumin and its derivatives are used to understand whether they inhibit the DNA nicking activity of Aβ(1-42). Also two plants *C.crista* and *C.asiatica* which showed antioxidant and anti-inflammatory activities, we tested whether they inhibit Aβ(1-42). Induced DNA nicking activity [See chapter 4A]. The curcumin and its derivatives could not inhibit the DNA nicking activity of Aβ(1-42). The aqueous leaf extracts of *C.crista* and *C. asiatica* also could not prevent the DNA nicking activity of Aβ(1-42). The CG and VG also bind differentially and change the secondary conformation

of Aβ(1-11), Aβ(1-28) and Aβ(25-35). This data clearly indicate that Curcumin, and plant extracts are potent in inhibiting oxidative stress and its mediated DNA damage. But our studies clearly indicate that these compounds could not prevent DNA nicking activity of Aβ(1-42). This data insights newer understanding in targeting drug discovery molecules against novel biochemical targets in neurodegeneration.

CHAPTER 3B

Aβ (1-42) induced MRI changes in aged rabbit brain resembles MRI changes in Alzheimer's disease brain

Chapter 3B Aβ(1-42) induced MRI changes in aged rabbit brain resembles MRI changes in AD brain

3B.1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in elderly people [Hardy and Higgins 1992]. AD is structurally characterized by brain atrophy due to loss of neurons [Vemuri *et al*., 2009]. Among many risk factors implicated in the cause of AD, Amyloid beta (Aβ) is the key factor implicated in AD [Selkoe, 2001]. The reasons for neuronal cell death in AD is still not clear Gupta *et al*., 2006]. Further, there are no reliable biomarkers for AD [Georganopoulou *et al*., 2006]. It has been attributed that CSF and plasma Aβmay be as biomarker and many researchers even try to correlate the Aβ versus brain atrophy. Recent studies clearly indicated that MRI changes in brain may support as good imaging biomarker for early diagnostic of AD [Kantarci, 2005; Rabinovici and Jagust, 2009]. But still scientists are debating on this relation [Rombouts *et al*., 2005]. The MRI measures the change in whole brain atrophy and different regional atrophy of the brain [Jack Jr *et al.,* 2005; Jack Jr *et al.,* 2004; Thodberg, 2003]. Studies involving AD patients have shown early involvement of medial temporal lobe structures such as entorhinal cortex and hippocampus in the disease initiation [Barnes *et al*., 2004; Lerch *et al*., 2005]. The decrease in the hippocampal volume is associated to severity of the AD [Jack Jr *et al*., 2004]. The study of Archer *et al.* [2006] reported that there is a positive correlation between amyloid load and cerebral atrophy in AD. Further studies indicated that the Aβ deposition load is found to have correlation with the degree of neuronal damage and cognitive deficits [Davies *et al*., 1988; Mann *et al*., 1985]. The microinjection of Aβ into cortex, hippocampus or amygdala has been reported to produce neuron loss and cholinergic degeneration [Chen *et al*., 1996]. However there is no animal model to mimic the total AD pathology [Bharathi *et al*., 2006]. Recently, Ramesh *et al*. [2010, Chapter 3A] has reported that intracisternal injection of Aβ (1-42) to aged rabbits has significantly altered the DNA conformation in frontal cortex, hippocampus and midbrain. The altered DNA conformation is C-type, π and ψ conformation in frontal cortex, hippocampus and midbrain respectively. However control rabbit brain regions showed B-DNA conformation. The ψ-conformation mimics the Z-DNA conformation [Suram *et al*., 2002]. The Z-DNA conformation is found in the hippocampus region of

the severe AD brain [Suram *et al*., 2002]. Also Aβ injection also altered DNA stability in brain regions. But these changes do not correlate with the changes in the rabbit brain atrophy. Ramesh *et al*. [2010] hypothesized that 25 days of Aβ injection is not sufficient to induce the brain atrophy though there are significant DNA instability and DNA conformation changes.

The present study is planned to investigate the effect of intracisternal injection of Aβ(1-42) on aged rabbit brain for 45 days. The brain structural changes in terms of thickness of frontal lobe, hippocampus, temporal lobe, midbrain and volume of lateral ventricle are analyzed. Additionally, the MRI data of rabbits is compared with AD patients MRI. This study will give an insight in understanding the role of Aβ(1-42) in inducing the brain atrophy in AD.

3B.2. Materials and methods

Aβ(1-42) was purchased from BZ Biolab Limited, USA. Saline was purchased from local medical store.

3B.2. 1.Animal protocol:

All animal procedures were carried out in accordance with Indian National Science Academy (India) animal protocol guidelines and rules framed by J.S.S. Animal Ethical Committee, J.S.S. Medical College, and Mysore, India. All the animals were housed in JSS Animal house in separate stainless steel cages. Ten New Zealand white aged rabbits (4.0 yrs old) received intracisternal injections of 100μL of normal saline (control) and another batch of ten aged rabbits received 100μL of (1mg/mL) Aβ(1-42) in saline. The injections were carried out under calmpose anesthesia, according to the modified protocol (Savory *et al*., 1999). The MRI was done before Aβ(1-42)/ saline injection and after 45 days of Aβ(1-42)/ saline injection. All the aged rabbits were subjected for MRI euthanized after 45 days. Aβ(1-42) injected animals have developed neurological symptoms including forward head tilting, hemiplegic gait, loss of appetite, isolation behavior, splaying of extremities and paralysis. These behavioral changes were the results of observations made on regular basis of every day from date of injection. The control animals did not display any behavioral disturbances.

3B.2. 2.MRI protocol for scanning aged rabbit brain

The rabbit's brains were imaged using MRI before injecting the Aβ(1-42). MRI images of aged rabbits injected Aβ(1-42) were taken after 45 days of injection. Scanning was done on Siemens Aavanto 1.5 T MRI scanner. Animal head was scanned using small flex coil for better positioning of the head in centre of the magnetic field so as to obtain better signal noise ratio. Animals were under the influence of diazepam (10mg) for about 20 minutes, which was sufficient to complete the MRI protocol. Aged rabbits were scanned in prone position. Images were acquired in coronal plane using T1, T2 and 3D gradient Echo Sequences. T2 sequences were obtained by using TR3500ms, TE79 ms, slice thickness of 3mm, and FOV 100mm. T1 sequences were obtained by following protocol TR-613ms, TE 11ms, 256X256 MATRIX FOV 100mm and slice thickness of 2mm. 3D GRE sequences were obtained by following protocol-TR9.5ms TE-4.76ms FOV-100ms and slice thickness of 1mm. The comparison of control animals with treated animals were done with the aim of demonstrating any structural change in the brain parenchyma which points towards loss brain volume. The thickness of frontal lobe, hippocampus, midbrain, temporal lobe and lateral ventricle was using system-attached software in MRI machine. Measurements were obtained for T2 Weighted images.

3B.2. 3. MRI protocol for scanning normal and human AD brain

5 normal and 5 AD patients were selected for the present investigation. The AD patients were diagnosed by NIH protocol by Psychiatry Professor of JSS Medical College, Mysore. MRI was done on all the above persons.

Multiplanar, multisequence MRI was done on Siemens Avanto 1.5T system using following protocol. Axial –T1, T2 and FLAIR (Fluid Attenuated inversion recovery). Sagittal –T1, Coronal –T2, 3D -gradient echo. Susceptibility weighted imaging –in axial plane, for T1- TR 550 ms and TE of 8.7 ms was used for T2 –TR of 5000 ms and TE of 118 ms was used for FLAIR sequence-TR was 9000 and TE was102 ms FOV for all sequence was 230mm. 5mm slice thickness was employed for all sequences, other than gradient sequence. 3D Gradient sequence was done in all patients in coronal plane with slice thickness of 1mm. All patients were co operative

and did not need any sedation. Typical total scan time for entire study was about 20 minutes.

3B.2.4.Statistical Analysis: The thickness and volume measurement data was analyzed using student t test in origin 6.0.3B.3.

3B.3.Results

3B.3.1. MRI study of aged rabbits with or without Aβ(1-42) injection The MRI is done for aged rabbits injected with Aβ(1-42) / saline. The results on the effect of Aβ(1-42) on thickness of different regions of aged rabbit brain frontal lobe, hippocampus, and midbrain is given in table1 and temporal lobe and lateral ventricle is given in table 2. Frontal lobe: There is a significant ($p<0.05$) decrease in the thickness of frontal lobe in case of Aβ(1-42) injected rabbit brain (both left and right) compared to saline(Table 3B.1). This indicates that there is a loss of neurons in the frontal lobe as may be affected by Aβ(1-42). Hippocampus: There is a significant ($p<0.05$) decrease in thickness of both left and right hippocampus of Aβ(1-42) injected rabbit brain compared to saline injected controls. Temporal lobe: Temporal lobe thickness data analyzed showed significant ($p<0.05$) reduction in the thickness of both control rabbit brain. Midbrain: The analysis of brain scans of midbrain for thickness of rabbit brain injected with Aβ(1-42) showed a significant reduction compared to saline injected controls. Lateral ventricle: The lateral ventricle thickness analysis evidenced an significant increase in the ventricular volume in the case of rabbit brain injected with Aβ(1-42) compared to saline injected control rabbits (fig.3B.3.1; Table 3B.2). This indicates the dilation of the ventricle, which is prominent feature of AD. The above results are in correlation with the structural changes as shown in MRI of AD patients (Fig3B.3. 2). For the first time we report significant structural changes as induced by Aβ(1-42) in aged rabbit brain. These results further validate the aged rabbits injected with Aβ(1-42) as a suitable animal model in studying the AD pathophysiology.

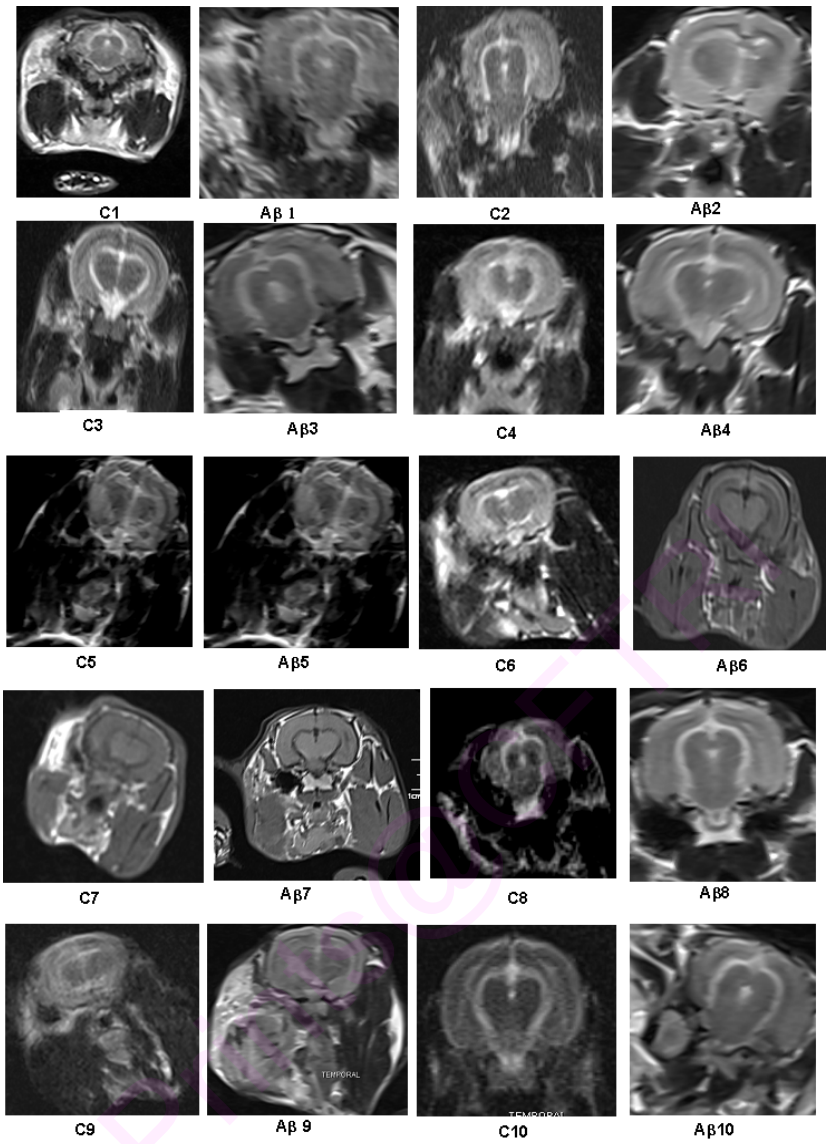


Fig 3B.3.1. Representative photos of coronal T1 brain slices except C6-C7 which ar T2 images. Figs C1–C10 are control rabbits. Figs Aβ(1–10 shows T1 brain slices except Aβ7, which is T2 image of the rabbits injected with Aβ(42). The image analysis is done by comparing the anatomy of control scans with scans obtained after injection of Aβ(1-42). The rabbits injected with Aβ(1-42) show significant changes in the frontal lobe, hippocampus, midbrain, temporal lobe size and lateral ventricle volume.

Table: 3B.1. The changes in the thickness of (mm) of different brain regions (Frontal lobe, hippocampus and midbrain) of Aβ(1-42) injected and control rabbits. * $p < 0.05$

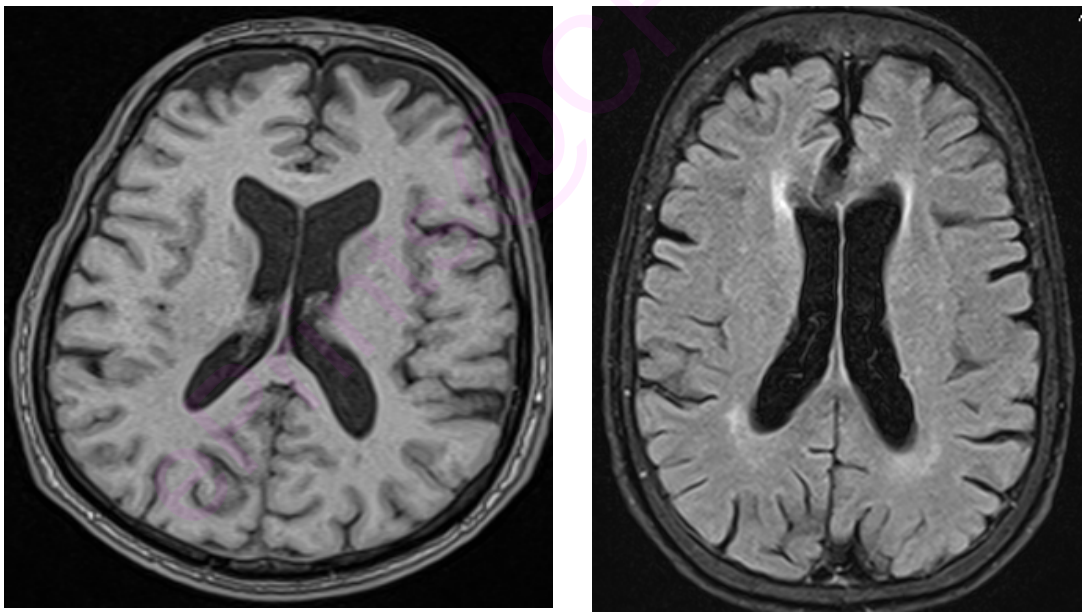
		FL/L		FL/R		Hc/L		Hc/R		Midbrain
Sl No	Aβ	C	Aβ	C	Aβ	C	Aβ	C	Aβ	C
1	2.6*	2.8	2.5*	2.6	1.7*	3.5	1.9*	2.9	3*	3.9
2	2.2*	3.2	2.3*	3.2	1.8*	3.2	1.8*	3.5	3.6*	4
3	2.5*	2.7	2.6*	3.2	1.8*	2.2	2*	2.2	3.4*	2.9
4	2.1*	2.8	2.3*	2.9	1.9*	3.2	2*	3.2	4.4*	4.6
5	2.1*	3.7	2.2*	3.3	2.3*	2.5	2.2*	2.3	4.3*	2
6	2.2*	2.6	1.9*	2.7	2.2*	2.3	1.6*	2.2	4*	4.4
7	1.9*	2.3	2.9*	2.3	1.6*	1.8	1.7*	1.8	4.1*	4.4
8	2.1*	2.3	2.4*	2.5	2*	2.4	2.2*	2.3	3.1*	3.8
9	2*	2.5	2.3*	2.6	1.9*	2.2	2*	2.1	4.2*	4.8
10	2.3*	2.3	2.2*	2.3	2.1*	2.7	2.3*	2.7	3.5*	4

Table: 3B. 2. The changes in the thickness of (mm) of different brain regions (Temporal lobe and lateral ventricle) of Aβ(1-42) injected and control rabbits. *p<0.05

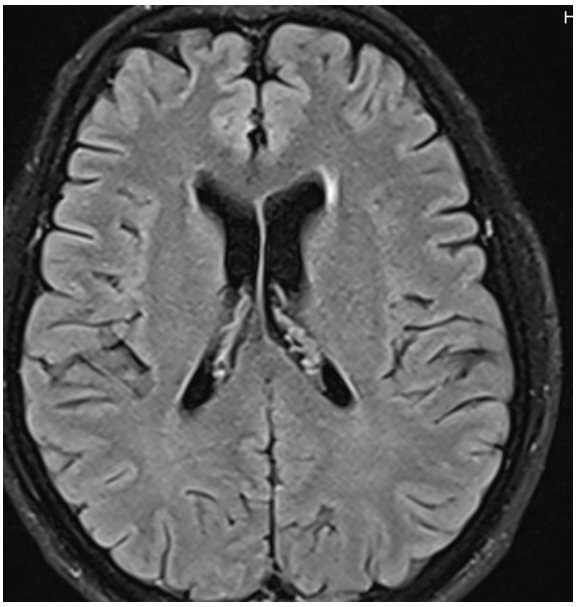
		TL/L		TL/R		LV/L		LV/R
Sl No	**Aβ**	**C**	**Aβ**	**C**	**Aβ**	**C**	**Aβ**	**C**
1	4.3*	6	4.4*	6.1	1.8*	1.7	2.2*	1.8
2	4.3*	6.2	5.4*	6.9	2*	1.4	1.9*	1.9
3	4.3*	6	5.4*	6.4	2.2*	1.4	2.4*	1.7
4	4.3*	5.7	5.6*	5.3	2*	2	2*	1.9
5	5.2*	5.4	5.6*	5.4	1.8*	1.6	1.9*	1.7
6	3.8*	5.2	3.9*	5.6	1.5*	1.2	1.9*	1.1
7	4.3*	4.9	4.6*	5.1	1.9*	0.9	1.5*	1
8	6.1*	6.7	5.5*	6.6	1.7*	1.1	2*	1
9	5.2*	6.4	5.1*	6.6	1.6*	1	1.4*	1.2
10	5.4*	5.3	5.2*	5.4	1.6*	1.2	1.8*	1.4

3B.3.2. MRI study of human AD and normal cases

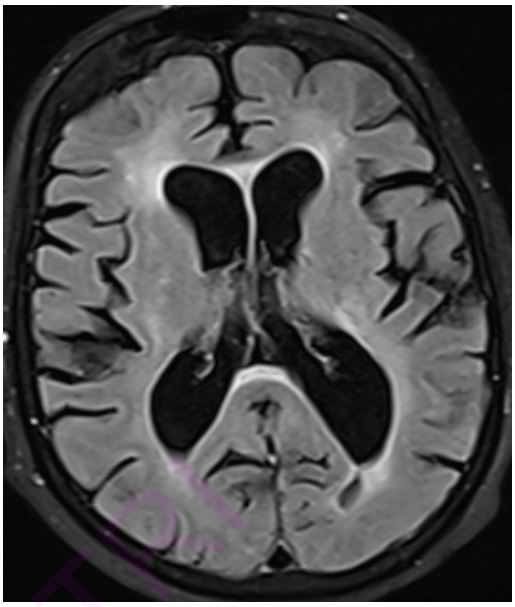
MRI study was conducted for 5 normal volunteers examined by neurologist confirming that they were not having any mental illness problems. The 5 AD cases were recruited from JSS hospital Mysore, after clear diagnosis by a neuropsychiatrist. The MRI is done for both normal and AD cases using 1.5 T MRI machine at Vikram hospital, Mysore. The thickness of frontal lobe, hippocampus, midbrain, temporal lobe, and lateral ventricle volume was analyzed using a inbuilt software in the MRI machine. The results showed that there is a significant reduction in the thickness of the frontal lobe and hippocampus in AD compared to normal. However there is no significant reduction in the thickness of midbrain, temporal lobe and lateral ventricle volume in AD compared to normal (fig.3B.3.2; Table 3B.3 and 4).



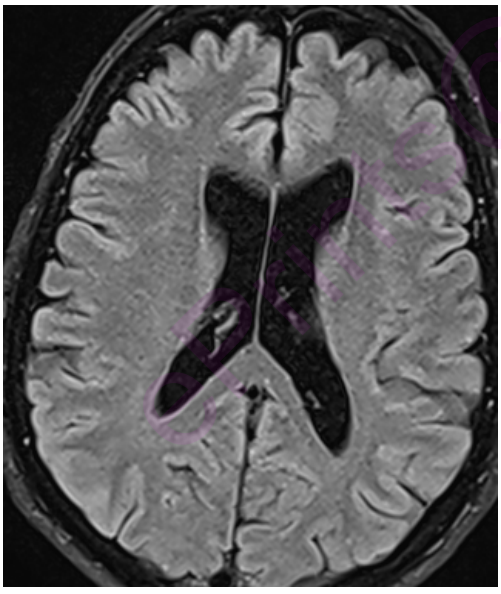
Normal 1 **AD 1**



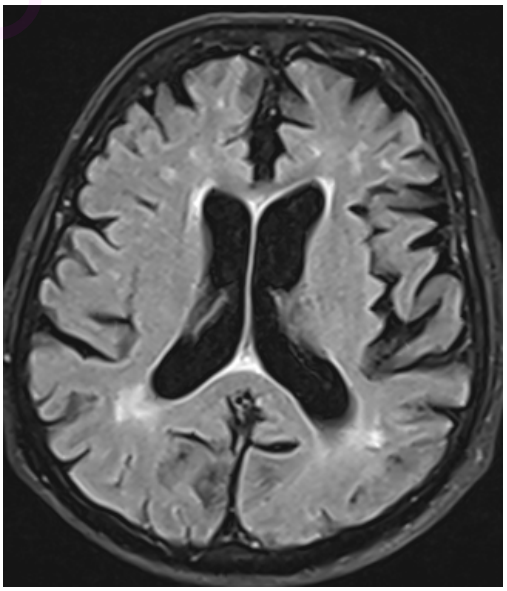
Normal 2



AD2



Normal 3



AD3

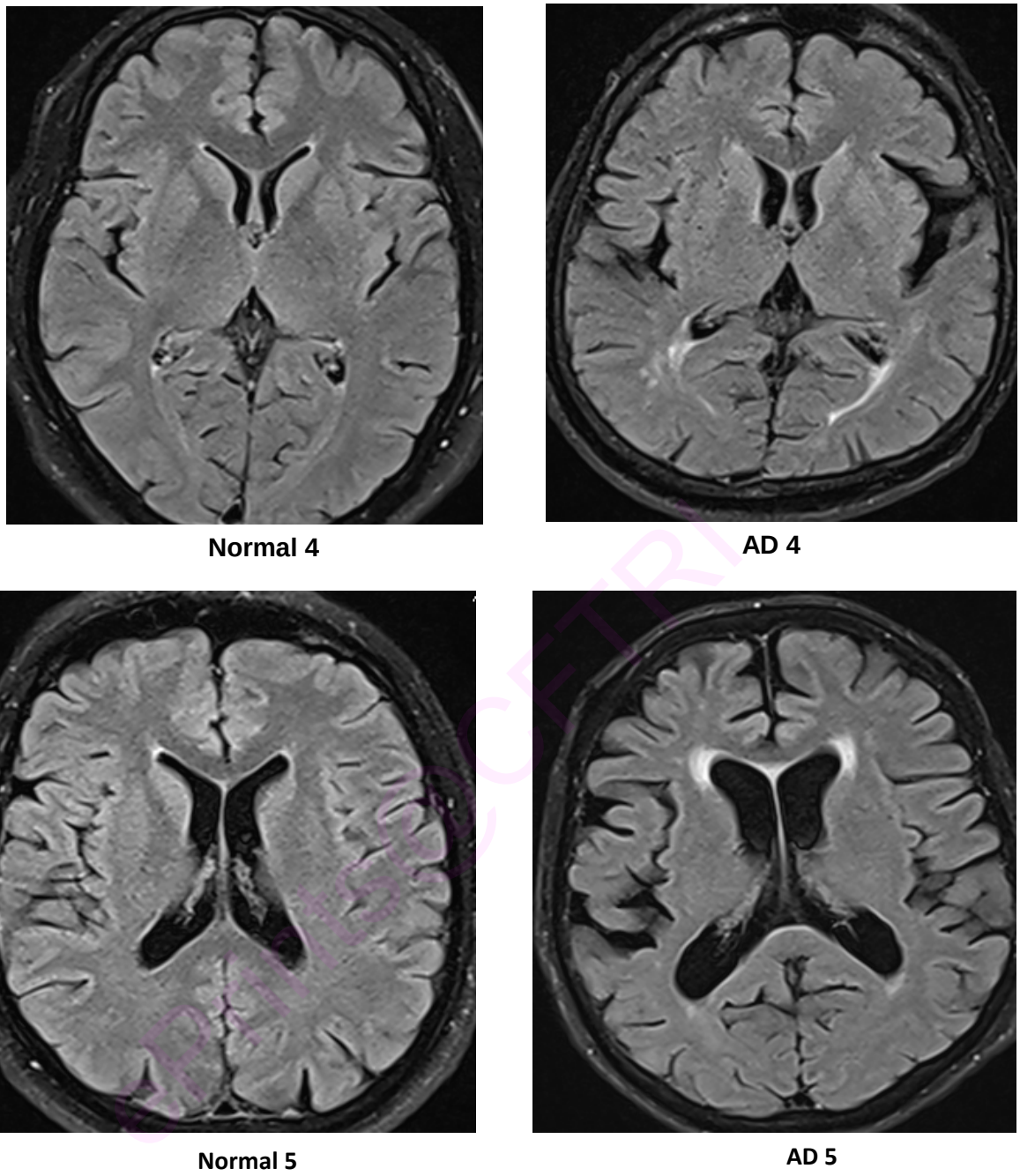


Fig 3B.3.2. Representative photos of coronal T1 brain slices of 5 normal brain and 5 AD brain. The image analysis is done by comparing the anatomy of normal brain scans with AD brain. The AD brain show significant changes in the frontal lobe, hippocampus, midbrain, temporal lobe size and lateral ventricle volume compared to normal.

Table 3B.3 The changes in the thickness (mm) of healthy and AD brain regions (Frontal lobe, Hippocampus and midbrain) * p< 0.05

	FL/L		**FL/R**		**Hc/L**		**Hc/R**		**Midbrain**	
	Healthy	**AD**	**Healthy**	**AD**	**Healthy**	**AD**	**Healthy**	**AD**	**Healthy**	**AD**
1	46.3	39.5*	46.7	42*	16.7	20.7*	17.9	8.6*	26.2	11.2
2	44	42.1*	46.3	41.8*	19.7	20.7*	18.5	15.2*	23.4	18.3
3	44.7	40.7*	48.3	37*	16.9	17.6*	16.4	11.7*	22.4	12.8
4	45.9	41.8*	46.9	39.9*	20.2	21.1*	18.1	16.6*	22.1	12.6
5	49.4	42.3*	47.8	40.9*	17.9	16.6*	17.2	15.1*	20	13.4

Table 3B.4 The changes in the thickness of (mm) healthy and AD brain regions (Temporal lobe and lateral ventricle)

TL/L		**TL/R**		**LV/L**		**LV/R**	
Healthy	**AD**	**Healthy**	**AD**	**Healthy**	**AD**	**Healthy**	**AD**
44.9	34	44.3	34.5	6.7	8.6	5.9	8.6
44.4	39.9	43.1	41.1	9.5	8.8	9.1	9
44.7	31.4	41.7	33.7	12.6	13.2	11.2	11.9
46.3	44.5	47.2	40.9	10.2	12.4	10	11.8
48.5	43.2	48.8	41.9	7.4	12.8	7.4	13.1

Discussion

Alzheimer's disease can be characterized by significant reduction in the structural integrity of brain. The neuronal cell loss followed by brain atrophy is the hall mark feature in AD. But the etiology and pathology of AD is still complex in nature [Perrin *et al*., 2009]. The amyloid beta (Aβ) is attributed as a key factor implicated in AD [Selkoe, 2001]. The mechanisms of cell death in AD are still not clear (Gupta *et al*., 2006]. Further, there are no reliable biomarkers for AD for early diagnosis [Georganopoulou *et al*., 2006]. It has been shown that CSF and plasma Aβ may be act like as biomarker and many researchers even try to correlate the Aβ levels versus brain atrophy [Galasko and Montine, 2010]. Recent studies clearly indicated that MRI changes in brain may support as good imaging biomarker for early diagnostic of AD [Kantarci, 2005; Rabinovici and Jagust, 2009]. But still scientists are debating on this relation [Rombouts *et al*., 2005]. The present proves few insights in this direction. The study by Price and Calderwood, (1992) reported that the levels of Aβ(40)/Aβ(1-42) in AD brain correlates with the frontal lobe atrophy as revealed by MRI. There is also report showing a positive correlation between the Aβ load and the reduction in brain volume and atrophy in AD patients [Archer *et al*., 2006]. Further, Fox *et al*. [2005] showed that immunization of AD patients with anti– Aβ (1-42)(AN1792/ QS-21) antibody reduced the loss of volume in cerebral region, indicating a relation between Aβ load to neuronal loss. Engler *et al*., [2006] has conducted a two year follow up study of amyloid deposition in relation to brain atrophy in AD patients. The results suggest that amyloid load is high and stable throughout two years. But there is a decline in regional cerebral metabolic rate for glucose and cognitive function [Engler *et al*., 2006]. This indicates that amyloid deposition in AD patients is a dynamic process and reaches equilibrium very early in the course of AD. Recently Sluimer *et al.* [2009] reported the progression of atrophy in AD patients. The study comprised of 64 AD patients, 44 with MCI and 34 controls. They subjected volunteers for serial MRI with time interval of 1.5 years. The results showed that a severe atrophy change in medial temporal lobe in MCI which is comparable to atrophy in AD patients. Additionally, atrophy rates are also increased in frontal lobe,

parietal and occipital lobe. In case of AD, atrophy rates are higher in extramedial temporal lobe and there is an increased atrophy changes in parietal, frontal, insular and occipital lobe. In the case of non-demented controls, medial temporal lobe atrophy is a good indicator of progressing towards AD. This indicates that though medial temporal lobe is affected very early in AD, over a period of progression of AD it spreads throughout the brain regions. The brain atrophy in AD initiates mainly at medial temporal region and fusiform gyrus. This atrophy can be seen 3 y before the diagnosis for AD. As the disease progresses, atrophy spreads to posterior temporal, parietal and frontal lobes. These atrophy changes are also seems to correlate with the neurofibrillary tangle distribution in AD [Whitewell, 2010]. The atrophy of brain is due to the loss of neurons. Atrophy can be described as widening of the sulci and shrinkage of gyri. The consequence of loss of neurons is the significant loss of brain weight [Whitewell, 2010].

To understand the mechanism of regional brain atrophy changes, we need to have suitable animal model. The intracerebroventricular infusion of Aβ(1-42) in rats resulted in progressive loss of brain functions such as behavioral, histological and neurochemical changes [Nakamura *et al*., 2001]. The MRI study on transgenic mice over expressing both APP and SOD or APP or SOD alone showed significant atrophy changes in different regions of brain. In case of double APP/SOD1 transgenic mice, the volume of hippocampus, entorhinal cortex and cingulated cortex reduced from 8 to 25%. But in the case of transgenic mice over -expressing SOD1there is a altered brain atrophy in cortex regions of cingulate, retrosplenial and temporoparietal cortex but there is no reduction in atrophy change in hippocampus atrophy. The transgenic mice over expressing APP alone do not show any changes in the brain atrophy compared to control [Borg and Chereul, 2008]. In an effort to develop animal model that mimic AD, we selected aged rabbit. The aged rabbits are more susceptible for developing AD like pathological features (Bharathi *et al*., 2006; Savory *et al*., 1999). The Aβ(1-42) is injected to aged rabbit through intracisternal route and allowed for 25 days. The results showed that Aβ do not induce significant structural changes in aged rabbit brain [Chapter 3A]. Since the process of neurodegeneration itself is a slow

process and may take long duration to show the gross changes in the brain structure. In the present study we injected Aβ(1-42) to aged rabbits and allowed for 45 days. The MRI results showed a significant reduction in thickness of frontal lobe, hippocampus, midbrain, temporal lobe and increase in the lateral ventricle volume. We also conducted MRI study on 5 AD and 5 normal cases. We also analyzed the thicknesses of frontal lobe, hippocampus, midbrain, temporal lobe and lateral ventricle lobe. We found significant reduction in thickness of the frontal lobe and the hippocampus. However, no significant reduction in the thickness of midbrain, temporal lobe and no significant increase in the lateral ventricle volume are observed compared to normal. The results of brain atrophy changes between rabbit brain and human AD brain correlation is established for frontal lobe and hippocampus regions. While, other regions such as midbrain, temporal lobe, lateral ventricle do not show correlation to rabbit brain atrophy changes in the corresponding regions.

The understanding of the changes in brain atrophy itself is complicated. Scahill *et al*. [2002] reported a significant expansion in lateral ventricle in mild AD, moderate AD and presymptomatic individuals. Additionally, there is a significant volume loss of hippocampus, precuneus, anterior frontal lobe cortices, and posterior part of cingulated gyrus in mild AD. The expansion of CSF spaces would correlate with total brain volume loss, which contributes to global atrophy. To determine the either atrophy or volume loss in different brain regions, one should judge which region to be scanned which may leads to biass in the measurement. The statistical parametric mapping is an automated technique for performing differences among different subgroups in AD such as mild, moderate AD, and AD. The statistical parametric mapping is also referred as voxel based morphometry [Ashburner and Friston, 2000]. There is a significant loss of temporal lobe volume in AD compared to normal. The level of atrophy of temporal lobe in AD is similar to mild AD [Teipel *et al*. 2006]. Clinically, there is a narrow difference between AD and fronto-temporal dementia. So, achieve clear cut diagnosis there is a need to have tool where it can differentiate the finer details. The fully automated freely available software "Free surfer" can be used to measure the cortical thickness. The study comprised on 28 confirmed AD, 11 amnestic type, 17 atypical AD like, 23 confirmed FTD and 25 healthy subjects

showed significant results. All the groups showed reduced cortical thickness in medial temporal lobe, posterior cingulate gyrus, precuneus, posterior parietal lobe and frontal lobe compared to controls [Lehman *et al*., 2010]. The reduced cortical thickness in anterior frontal lobe and frontal lobe is specific marker for fronto-temporal dementia. The reduction in thickness of posterior cingulate gyrus is characteristic of AD with or without typical or atypical features. The atrophy changes are attributed to the loss of neurons and the neuronal tissue. The loss of brain tissue makes the accumulation of CSF in the spaces. The cause of neuronal loss and subsequent changes in atrophy and reduction in brain volume is not understood completely. Recent reports suggest that there is a correlation between the amyloid load and brain atrophy in presymptomatic stage of AD [Chetetat *et al*., 2010; Frisone *et al*., 2009]. But there was no significant correlation between the amyloid load and atrophy in mild cognitive impairment and AD. This indicates the effect of amyloid in neurodegeneration. The brain regional accumulation of amyloid and its corresponding toxicity in terms of loss of neurons has a great impact in understanding the process of neurodegeneration. In this direction, Frisoni *et al*. [2009] found more amyloid in frontal, parietal and posterior cingulate gyrus cortical areas. However, there is a significant correlation between the amyloid load and higher atrophy changes in hippocampus and amygdale, while no correlation is obtained for frontal, temporal, posterior cingulate/ retrosplenal, insular and caudate region. In our study, we do not find the significant changes in the thickness of temporal lobe and midbrain. Though we do not quantify the amyloid load in different regions but we found significant reduction thickness of frontal lobe and hippocampus in AD compared to age matched healthy subjects. Our MRI studies with aged rabbits injected with Aβ(1-42) showed correlation to human AD MRI a in two important regions such as hippocampus and in temporal loge atrophy. In the literature, AD cases with amyloid load correlated to brain atrophy of that particular region [Frisoni *et al*., 2009]. This data shows that bet-amyloid is directly causes toxicity in vivo by causing neuronal cell death and consequently brain atrophy. Since an Alzheimer's disease animal model which depicts brain atrophy changes in different brain regions is essential to understand regional toxicity of beta-amyloid. So our animal model will be useful in testing molecules that reverse the toxicity of beta-amyloid by restoring brain atrophy changes. Our study for the first time showed that intracisternal injection of Abeta showed significant reduction in the thickness of

frontal lobe, hippocampus, temporal lobe, midbrain and increase in the thickness of lateral ventricle. We also evidenced the correlation between aged rabbits injected with Aβ(1-42) and human AD interms of the changes in thickness of hippocampus and frontal lobe. That is significant reduction in the thickness of frontal lobe and hippocampus are observed in aged rabbits and in human AD brains. However, no correlation is observed for midbrain, temporal lobe thickness and lateral ventricle volume. This data would aid in understanding the role of Abeta load in the early onset of AD prior the expression of symptoms.

CHAPTER 4A

Antioxidant and anti-inflammatory activities of leaf extracts of C. crista and C. asiatica

Chapter 4A Anti-oxidant and anti-inflammatory activities of *C.crista* and *C.asiatica*

4A. 1. Introduction

The oxidative stress and inflammation have been implicated in neurodegenerative disorders like AD, PD etc [Patten *et al*., 2010; Reynolds *et al*., 2007]. The generation of reactive oxygen species, which are toxic, is a part of normal metabolism in biological system. The balance between the production of reactive oxygen species and anti-oxidants is essential in biological system to prevent adverse effects of oxidative stress. The imbalance between reactive oxygen species and antioxidants in human body leads to oxidative stress [Houstis *et al*., 2006]. The oxidative stress has detrimental effects on central nervous system [Reynolds *et al*., 2007]. The brain is more prone for oxidative stress because of (i) brain is rich in easily oxidizable unsaturated fatty acids; (ii) brain requires more oxygen per unit weight (20% of total oxygen requirement in human beings); (iii) brain is also rich in iron and ascorbate which are key players in oxidation; and (iv) brain is deficient in antioxidants. The free radicals thus generated are known to attack macromolecules such as DNA, proteins, lipids and carbohydrates. This leads to either onset or acceleration of degenerative disorders [Durackova, 2009; Ishizaki *et al*., 1996; Kehrer, 1993]. The free radical induced damage to biomolecules in AD is characterized by the appearance of modified nucleic acids, such as 8-hydroxyl-2-deoxyguanosine (8-OHG). Also, DNA fragmentation and DNA breakage has been reported in AD patients [Nunomura *et al*., 1999]. There are reports on the oxidative modification of proteins such as protein carbonyls and nitration of the tyrosine residues [Smith *et al*., 1997]. Other proteins that are modified by oxidative mechanisms include enzymes of oxidative phosphorylation and glycolysis. The lipid component in the brain is subjected to oxidative stress and results in the formation of thiobarbituric acid reactive substances, malondialdehyde, 4-hydroxy-2-transnonenal and isoprostane [Butterfield *et al*., 2001]. The carbohydrates molecules such as sugars are also modified by oxidative stress and it is evidenced by the increased levels of glycated end products in AD patients [Vitek *et al*., 1994]. Overall, the oxidative modification of biomolecules leads to the imbalance in the metabolic activities in AD [Sultana *et al*., 2009]. The cross linking in nucleic acid, and protein will make the removal of cross linked products difficult

which may accelerate the progression of AD. The accumulating evidences on the role of oxidative stress suggest that it may be an early event in the onset of AD [Pratico *et al*., 2001; Smith *et al*., 1997, Chauhan and Chauhan, 2006; Lahiri *et al*., 2008; Gua *et al*., 2008]. The evidence for early involvement of oxidative stress is by the appearance of oxidative modification and oxidative damage even before the appearance of the NFT and amyloid deposits. Additionally, accumulation of 8-OHG and nitrotyrosine one decade before the accumulation of amyloidbeta in Down's syndrome at teens and twenties add support for the above concept. Though the generation of reactive oxygen species in AD is not clearly understood, the suggested mode of the generation of free radicals are: (i) direct generation by amyloidbeta; (ii) activation of microglia by amyloidbeta; and (iv) defects in electron transport chain etc. It was also suggested that reactive oxygen species interacts with the amyloidbeta protein to form senile plaques and generate more reactive free radicals and damage the cell membrane [Mattson and Godman, 1995; Chalimoniuk *et al*., 2007; Reddy and Beal, 2008; Sultana and Butterfield, 2010]

There is a growing evidence that suggest the mitochondrial dysfunction in AD has a significant role to understand neurodegeneration [Reddy *et al*., 2010; Schon and Area-Gomez, 2010; Dumont *et al*., 2010]. The mitochondrial dysfunction may be attributed to the abnormalities in mitochondrial genome [Davis *et al*., 1997] or deficiencies in key mitochondrial enzymes [Sorbi *et al* 1983; Sheu *et al* ., 1985; Blass *et al*., 1990; Parker *et al*., 1990; Du *et al*., 2008; Supnet and Bezprozvanny, 2010]. The conversion of toxic $0^{\bullet-}_2$ is mediated by mitochondrial superoxide dismutase-1. This enzyme is found to be decreased in AD [Esposito *et al*., 2006]. The reduced function of mitochondria in AD is attributed to accumulation of APP in mitochondria [Anandatheerthavarada *et al*., 2003; Devi *et al*., 2006; Keil *et al*., 2004; Park *et al*., 2006, Hoye *et al*., 2008; Devi and Anandatheerthavarada, 2010; Pavlov *et al*., 2009]. Additionally, APP695 accumulation in human cortical neuronal cell line is also linked to the reduced membrane potential,decreased ATP generation and reduced cytochrome C activity [Ananthatheerthavarad *et al*., 2003]. Also, amyloid deposition is found in mitochondria of human AD brain [Lustbader *et al*., 2004; Devi *et al*., 2006] and in Aβ over expressing transgenic mice [Caspersen *et al*., 2005; Manczak *et*

al., 2006; Crouch *et al*., 2005]. There is a evidence linking between Aβ and mitochondrial dysfunction [Chen and Yan, 2007; Marques *et al*., 2009]. Both *in vitro* and *in vivo* studies revealed that Aβin the mitochondria is associated with increased levels of hydrogen peroxide, decreased cytochrome c activity and higher levels of carbonylated proteins [Manczak *et al*., 2006, Long *et al*., 2009; Kadenbach *et al*., 2009; Mai *et al*., 2010].

The defense system of the human body against oxidative stress induced impairments incudes antioxidant enzymes and non-enzymatic antioxidant proteins [Butterfield *et al*., 2002]. The enzymatic defense includes superoxide dismutase, catalase and glutathione reductase [Calabrese *et al*., 2006]. The non-enzymatic defense molecules include reduced glutathione, albumin, ferritin and ceruloplasmin. These inbuilt defense system are reduced in AD [Uttara *et al*., 2009]. Hence are trying to supplement natural antioxidants. There are reports on natural source being used as antioxidants in animal models to test their efficacy [Veurink *et al*., 2003; Sharma and Gupta, 2002; Perluigi *et al*., 2006; Zhao, 2009; Mancuso *et al*., 2007; Vingtdeux *et al*., 2008; Lleo *et al*., 2006; Aliev *et al*., 2008; Darvesh *et al*., 2010].

The inflammation is another responsible factor in AD and is presumed to be mediated through the cross talk among the amyloid, astrocytes and microglia [Kitazawa *et al*., 2004; Zotova *et al*., 2010]. These reactions lead to altered neuronal function and the inflammatory injury [Kitazawa *et al*., 2004]. The leukotriens and prostaglandins are the mediators of inflammatory response in the cell [Crooks and Stockely, 1998]. The biosynthesis of leukotriens is initiated by the activation of phospholipase A2 (PA2). PA2 in turn hydrolyzes membrane phospholipids to release arachidonic acid (AA). The 5-lipoxygenase hydrolyzes AA in calcium and ATP dependent reaction to leukotriens and prostaglandins. 5-lipoxygenase enzyme is important in the biosynthesis of leukotriens and its location is confined to polymorphonuclear leukocytes (PMNLs), monocytes, macrophages, mast cells and B-lymphocytes [Raghavendra *et al*., 2006]. The 5-lipoxygenases catalyze the addition of dioxygen at C-5 of AA to form 5-hydroperoxy eicosa tetetra enoic acid (5-HPETE). 5-HPETE will be metabolized to leukotriens, LTB4, LTC4, LTD4 and LTE4. These

leukotriens are implicated in rheumatoid arthritis, psoriasis and glomerulonephritis [Crooks and Stockely, 1998]. Since 5-lipoxygenase mediates a key step in the generation of inflammatory molecules, modulation of its activity have therapeutic implications [Manev *et al*., 2000; Ikonomovic *et al*., 2008]. For screening the compounds for anti-inflammatory actions, 5-lipoxygenase provides a good *in vitro* model.

Recently we have reported the effects of aqueous extracts of *C. crista* and *C. asiatica* in inhibiting the amyloid fibril formation and dis-aggregation of pre-formed fibrils [Ramesh *et al*., 2010; Ramesh *et al*., 2010 and also see Chapter 4B and C]. The results showed that *C. crista* not only inhibited the amyloid fibril formation but also could dis-aggregate the pre-formed fibrils [see Chapter 4B]. However, *C. asiatica* could not able to prevent the amyloid fibril formation and also could not dis-aggregate the pre-formed fibrils [Chapter 4C]. Since *C .crista* has shown promising anti-amyloidogenic property, we also want to test these extracts for the antioxidant and anti-inflammatory activities. The present study is undertaken to study the effects of the aqueous extracts of both *C. crista* and *C. asiatica* for their antioxidant and anti-inflammatory activities.

4A. 2. Material and methods

Materials: 1,1-Diphenyl-2-picrylhydrazyl (DPPH), adenosine triphosphate (ATP), dithreitol (DTT), arachidonic acid (AA), nordihydroguaiaretic acid (NDGA) from Sigma Chemical Co., MO, USA. Nicotin-amide adenine dinucleotide (reduced)-NADH, copper sulphate, Sodium potassium tartarate and phenzine methosulfate (PMS) are purchased from Hi-media, Mumbai, India. NitroBlue tetrazolium (NBT) is purchased from Sisco Research Laboratories, Mumbai, India. All other chemicals and solvents used are of analytical grade.

The detailed description of source of the plants, its taxonomic identification and preparation of the aqueous leaf extract is given in Chapter 4B and C.

4A. 2. 1. Phytochemical tests

The qualitative phytochemical tests were performed for establishing chemical profile of leaf aqueous extracts of *C. crista* and *C. asiatica* extracts. The following

phytochemocal tests were performed to detect various phytoconstituents present in extracts.

1.Detection of Alkaloids [Gibbs, 1974; Peach and Tracey, 1954; Trease *et al* 1978]

Solvent free extract (50mg) was stirred with few mL of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents as follows:

a. Mayer's test

To a few mL of filtrate, a drop or two of Mayer's reagent was added along the side of test tube. A white or creamy precipitate indicates the test as positive.

Mayer' reagent: Mercuric chloride (1.358g) was dissolved in 60mL of water and potassium iodide (5.0g) was dissolved in 10mL of water. The two solutions were mixed and made upto 100mL with water.

b. Wagner's test

To a few mL of filtrate, few drops of Wagner's reagent were added by side of the test tube. A reddish –brown precipitate confirms the test as positive.

Wagner's reagent:

Iodine (1.27g) and potassium iodide (2g) was dissolved in 5mL of water and made up to 100mL with distilled water.

c. Hager's test

To a few mL of filtrate, one or two mL of Hager's reagent (saturated aqueous solution of picric acid) was added. A prominent yellow precipitate indicates the test as positive.

d. Dragendorff's test

To a few mL of filtrate, one or two mL of Dragendorff's reagent was added. A prominent yellow precipitate indicates the test as positive.

Dragendorff's reagent:

Stock solution: Bismuth carbonate (5.2g) and sodium iodide (4g) were boiled for a few minutes with 50mL glacial acetic acid. After 12 hours, the precipitated sodium

acetate crystals were filtered off using sintered glass funnel. 40mL of clear red-brown filtrate was mixed with 160mL ethyl acetate and 1mL water and it was stored in amber-colored bottle.

Working solution: 10mL of stock solution was mixed with 20 mL of acetic acid and made up to 100mL with water.

2. Detection of carbohydrates

The extract (100mg) was dissolved in 5mL of water and filtered. The filtrate was subjected to the following tests.

a. Molish's test:

To two mL of filtrate, two drops of alcoholic solution of alpha-naphthol are added, the mixture was shaken well and one mL of concentrated sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicates the presence of carbohydrates.

b. Fehling's test

One mL of filtrate was boiled on water bath with one mL each of Fehling solutions A and B. A red precipitate indicates the presence of sugar.

Fehling's solution A: Copper sulphate (34.66g) was dissolved in distilled water and made up to 500mL using distilled water

Fehling's solution B: Potassium –sodium tartarate (173g) and sodium hydroxide (50g) was dissolved in water and made up to 500mL.

c. Barfoed's test

To one mL of filtrate, one mL of Barfoed's reagent was added and heated on a boiling water bath for two minutes. A red precipitate indicates the presence of sugar.

Barfoed's reagent:

Copper acetate, 30.5g was dissolved in 1.8mL of glacial acetic acid.

d.Benedict's test

To 0.5mL of filtrate, 0.5mL of Benedict's reagent was added. The mixture was heated on a boiling water bath for two minutes. A characteristic colored precipitate indicates the presence of sugar.

Benedict's reagent

Sodium citrate (173g) and sodium carbonate (100g) were dissolved in 800mL of distilled water and boiled to make it clear. Copper sulphate (17.3g) dissolved in 100mL-distilled water and added to above solution.

3. Detection of proteins and amino acids [Fisher, 1968]

The extract (100mg) was dissolved in 10mL of distilled water and filtered through Whatmann No.1 filter paper and the filtrate was subjected to test for proteins and aminoacids.

a. Millon's test [Rasch and Swift, 1960]

To two mL of filtrate, few drops of Millon's reagent were added. A white precipitate indicates the presence of proteins.

Millon's reagent: Mercury (1g) was dissolved in 9mL of fuming nitric acid. When the reaction was completed, equal volume of distilled water was added.

b.Biuret test

An aliquot of two mL of filtrate was treated with one drop of 2% copper sulphate solution. To this, one mL of ethanol (95%) was added, followed by excess of potassium hydroxide pellets. Pink color in the ethanolic layer indicates the presence of proteins.

c. Ninhydrin test [Yasuma and Ichikawa, 1953]

Two drops of ninhydrin solution (10mg of Ninhydrin in 200mL of acetone) were added to two mL of aqueous filtrate. A characteristic purple color indicates the presence of aminoacids.

4.Detection of phytosterols [Finar, 1986]

Libermann- Burchard's test:

The extract (50mg) was dissolved in 2mL acetic anhydride. To this, one or two drops of concentrated sulphuric acid were added slowly along the sides of the test tube. An array of color changes shows the presence of phytosterols.

5. Saponification test:

A few drops of 0.5 N alcoholic potassium hydroxide solutions were added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on a water bath for two hours. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

6. Detection of phenolic compounds and tannins

a. Ferric chloride test [Mace, 1963]

The extract (50mg) was dissolved in 5mL of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green color indicates the presence of phenolic compounds.

b. Alkaline reagent test:

An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids.

c. Magnesium and hydrochloric acid reduction [Harborne, 1998]

The extract (50mg) was dissolved in 5mL of alcohol and few fragments of magnesium ribbon and concentrated hydrochloric acid (drop wise) were added. If any pink to crimson color develops, presence of flavanol glycosides was inferred.

4A.2.2. High-pressure thin layer chromatography (HPTLC):

The aqueous leaf extract was subjected to HPTLC (CMAG TLC system) to map the chemical contents of the aqueous leaves extracts of *C. crista* and *C. asiatica*.

a. Selection of HPTLC plates. The pre-coated and pre-activated TLC plates (E. Merck No. 5548) of silica gel 60 $F_{254+366}$ with the support of aluminium sheets having thickness of 0.1mm and size 20x20 cm were cut into smaller size according to required dimensions.

b. Sample preparation: 100 mg aqueous leaf extracts of *C. crista* and *C. asiatica* was dissolved in 10mL of methanol and stirred. The contents were filtered and filtration was repeated to get clear solution. The sample volume was made upto to 10 mL using methanol. The solvent system used was ethyl acetate: formic acid: glacial acetic acid: water in the ratio: 100:11:11:26. Standard used was quercetin. The solvent system when wass changed when gallic acid was used as standard. The solvent system used was toluene: ethyl acetate: formic acid in the ratio; 4:5:1.

c. Application of sample. The extract sample was applied in the form of a band using CAMAG LINOMAT IV, an automatic sample application device, maintaining a band width 9 mm, space 9 mm, 15 sec $\mu L^{-1.}$ The quantity of sample applied was 5–10 μL.

d. HPTLC analysis. The following mobile phase was selected experimentally: formic acid: glacial acetic acid: water in the ratio: 100:11:11:26. The plates were developed by placing in pre-saturated tank (12 cm height) with mobile phase for 2 h. The plates were dried by evaporating the solvent either at room temperature or by spraying hot air by air dryer. After developing the TLC plate, it is scanned and recorded.

4A. 2. 3. Antioxidant assays

(i). DPPH radical scavenging assay

1,1-Diphenyl-2-picryl hydrazyl (DPPH) is a stable free radical that accepts an electron or hydrogen atom to become a stable 1,1-Diphenyl-2-picrylhydrazine molecules. The reduction of DPPH radical was determined by decrease in the absorbance at 517 nm. The antioxidant activity of leaf aqueous extracts of *C. crista and C. asiatica* and standard synthetic antioxidant ascorbic acid was measured in terms of hydrogen donating or radical scavenging ability [Brand -Williams *et al*., 1995]. Briefly 1 mL of 200 µM methanolic solution of DPPH was incubated with different concentrations of *C.crista* and *C.asiatica* extracts and standard ascorbic acid for 20 minutes at room temperature. At the end of incubation period, the absorbance is measured using a UV-Visible spectrophotometer at 517 nm. The percentage of scavenging or quenching of DPPH radicals (Q) by *C. crista, C. asiatica* and ascorbic were calculated using the following formula.

$$Q = 100\,(A0 - Ac)/A0$$

Where Ao is the absorbance of the control tube and Ac was the absorbance of the tube with 'c' concentration of sample. All the experiments were performed in triplicates.

(ii). Superoxide anion scavenging assay

Superoxide anion scavenging activity of *C. crista*, *C.asiatica* and ascorbic acid were determined by a modified method of Liu *et al*. [1997]. Superoxide radicals were generated in PMS-NADH system by oxidation of NADH, and assayed by the reduction of nitroblue tetrazolium (NBT) [Nishikimi *et al*., 1972]. The assay system consists of 100 µl each of 1.0 mM NBT, 3.0 mM NADH and 0.3 mM PMS and the final volume was adjusted to 1mL with 0.1 M phosphate buffer (pH 7.8), at ambient temperature. The reaction mixture (NBT and NADH) was incubated without or with

C. crista, *C. asiatica* and ascorbic acid extracts at room temperature for 2 minutes and the reaction was started by adding PMS. The absorbance at 560 nm was measured against blank samples for 3 minutes. Decrease in absorbance in the presence of *C. crista*, C. *asiatica* extracts and ascorbic acid indicates superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

Inhibition of superoxide generation (%) = ***[(A0-A1)/A0] x 100,***where, A0 was the absorbance of the control, and A1 was the absorbance in presence of extract.

(iii) Reducing potential: Potassium ferricyanide reducing method

The reductive potential of the *C. crista*, and *C. asiatica* extracts was determined according to the method of Oyaizu *et al* [1986]. Different concentrations of *C. crista* , and *C. asiatica* extracts in 0.5 mL of water were mixed with equal volumes of 0.2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide (K3Fe(CN)6). The mixture was incubated for 20 minutes at 50 ^{O}C. At the end of incubation, an equal volume of 10% trichloroacetic acid is added to the mixture and centrifuged at 3200 xg for 10 minutes. The supernatant was mixed with distilled water and 0.1% ferric chloride at 1:1:0.2 (v/v/v) and the absorbance were measured at 700 nm. An increase in the absorbance of the reaction mixture indicates the potential reducing power of the sample. Ascorbic acid was used as a standard for comparison.

4A. 2.4. Anti-inflammatory activity: 5-Lipoxygenase assay

4A.2.4.1. Isolation of 5-Lipoxygenase human polymorph nuclear leukocyte (PMNL): Human peripheral venous blood from healthy persons who have not under any medical prescription was collected in tube containing EDTA. PMNLs were isolated from blood by ficoll-Histopaque density gradient followed by hypotonic lysis of erythrocytes [Boyum, 1976]. All the procedures were performed at 4^{0}C. PMNLs were re-suspended in phosphate buffer saline and sonicated for 20-30 seconds at 20kHz to release the cytosolic 5-LO enzyme into solution. This solution was centrifuged at 100,000g for 30 minutes at 4^{0}C and the supernatant was used as a source of enzyme.

4A.2.4.2.Protein estimation:

Protein content in human polymorph nuclear leukocyte was estimated according to Lowry's method (Lowry *et al.,* 1951). To 1 mL of protein solution, 5 mL of reagent C containing a mixture of 2% sodium carbonate in 0.1 M sodium hydroxide and 0.5% copper sulphate in 1% sodium potassium tartarate is added. It was allowed to stand for 10 min at ambient temperature. To this, 0.5 mL of 1:1 diluted FC reagent is added, and kept at ambient temperature to develop the colour. The absorbance of the sample was read at 700 nm and the amount of protein present is determined by referring to the standard graph prepared by using bovine serum albumin.

4A.2.4.3. 5- lipoxygenase enzyme assay

5- lipoxygenase enzyme assay was performed using previously reported method [Aharony and Stein, 1986]. The enzyme reaction mixture contains 100mM pH 7.4, 50μM DTT, 200μM ATP, 300μM CaCl2, 150μM AA and 5μg enzyme. The aqueous leaf extracts of *C. crista* and *C. asiatica* were incubated with the enzyme for 2 minute prior to the addition of AA. The enzymatic reactions were carried out at room temperature. 5-lipoxygenase activity was measured as 50-HETE formed at 234nm using spectrophotometer (Shimadzu).

4A.2.5. Trace element analysis of *Caesalpinia crista* and *Centella asiatica* aqueous extract and whole leaf extract by Inductively Coupled Plasmon Atomic Emission Spectrometry (ICP-AES)

The trace element analysis was done by ICP-AES (JY70, Jobin Yvon, France). The analysis was made either by sequentially or simultaneous mode depending on the elements to be analyzed. All the dilutions were made with ultra pure Milli Q water (18MΩ) in dust free environment. For the optimization of the ICP-AES method, lines were selected and detection limits evaluated for each element. The lines were chosen for each element in a way to obtain minimum interferences from other elements. The wavelengths are used and detection limits of the elements were summarized in table 1. Quality control of the analyses was performed by analyzing a serum matrix matched multi-element synthetic standard reference material (Bovine liver 1577a) obtained from national bureau of standards, USA [Rajan *et al* 1998].

4A.2.5. Statistical analysis

The IC_{50} data was generated from the dose response curves. The data represented as % inhibition of DPPH and superoxide radical. The 5-5-lipoxygenase-enzyme activity inhibition was represented as % inhibition in presence of aqueous extracts of *C. crista* and *C. asiatica*. The values were given as mean± SEM of four individual samples.

Table 4A.1: Inductively coupled plasma atomic emission spectrometry: Wavelength and detection limits

Element	Wavelength (nm)	Detection limit	
		µg/ml	µmol/ml
Na	588.995	0.03	0.00130
K	766.49	0.06	0.00153
Se	196.090	0.075	0.00156
P	213.618	0.05	0.00162
Ca	393.366	0.002	0.00005
Mg	279.806	0.001	0.00004
Cu	224.7	0.002	0.00003
Zn	213.856	0.002	0.00003
Fe	259.94	0.005	0.00009
Mn	257.61	0.001	0.00002

Table 4A.2: Qualitative phytochemical screening of extracts of *C. crista* and *C. asiatica.*

SlNo	Photochemical test	*C. crista*	C. asiatica
1	**Alkaloids**		
A	**Mayer's test**	Negative	Negative
B	**Wagner's test**	Negative	Negative
C	**Hager's test**	Negative	Negative
D	**Dragendorff's test**	Negative	Negative
2	**Carbohydrates and Glycosides**		
A	**Molish's test**	Positive	Positive
B	**Fehlings test**	Positive	Positive
C	**Barfoed's test**	Positive	Positive
D	**Benedicts test**	Positive	Positive
3	**Proteins and amino acids**		
A	**Millon's test**	Positive	Positive
B	**Biuret test**	Negative	Negative
C	**Ninhydrin test**	Negative	Negative
4	**Phytosterol test**	Positive	Positive
5	**Saponin's test**	Positive	Positive
6	**Phenolic compounds and flavonoids**		
A	**Ferric chloride test**	Positive	Positive
B	**Magnesium and Hydrochloric**	Positive	Positive
C	**Alkaline test**	Positive	Positive

4A. 3. Results

4A. 3.1. Qualitative Phytochemical assay

The qualitative phytochemical test was performed to have information on chemical composition of the leaf aqueous extracts of *C. crista* and *C. asiatica* and characterize the compounds. This information will help biochemists to follow the right path in isolation and characterization of the compound from the crude extract. We subjected aqueous leaf extracts *of C.crista* and *C. asiatica* for phytobiochemical composition analysis. The results showed that both the aqueous extracts showed positive for carbohydrates, proteins, aminoacids, saponins, phenolics and flavonoids. However, both the extracts showed negative results for alkaloids (Table 2).

4A 3.2. Trace metal analysis by ICP-AES

Trace metals were analyzed in aqueous extracts and whole leaf of *C. crista* and *C. asiatica*. The concentration of the trace metals were expressed in parts per million (ppm) (aqueous extract is given table 4A. 3 and whole leaf extract is given in table 4A. 4).The trace metals such as iron, copper, zinc were higher in aqueous extract of *C. crista* compared to *C. asiatica.* However, in the whole leaf, the concentration of iron, copper and zinc was higher in *C. asiatica* compared to *C. crista*.

Table 4A. 3: Trace element analysis (ppm) of *Caesalpinia crista* and *Centella asiatica* aqueous extract by Inductively Coupled Plasma Atomic Emission Spectrometry.

SlNo	Element(ppm)	*Caesalpinia crista*	*Centella asiatica*
1	Ca	12,413	21,241
2	Cu	18	5
3	K	1,43,000	151,000
4	Mg	14,917	15,410
5	Mn	302	350
6	Fe	400	160
7	Na	4800	4200
8	P	10,586	5069
9	Se	0.9	<0.01

10	Zn	40	36

Table 4A.4:Trace element analysis (ppm) of *Caesalpinia crista* and *Centella asiatica* whole leaves by Inductively Coupled Plasma Atomic Emission Spectrometry

SlNo	Element (ppm)	*C. crista*	*C. asiatica*
1	Ca	9500	11650
2	Cu	5	30
3	K	6750	2850
4	Mg	2850	6000
5	Mn	124	65
6	Fe	100	400
7	Na	336	14850
8	P	1000	3180
9	Se	0.9	0.9
10	Zn	20	64

4A. 3.3. HPTLC mapping of phyto-biochemicals in *C.crista* and *C. asiatica*

The mapping of constituents of crude aqueous leaf extract was done using HPTLC. The results showed that gallic acid was present in *C. crista* and absent in *C. asiatica* where as quercetin was present both in *C. crista* and C.*asiatica* (Fig 4A.1a, Fig 4A.1b, Fig 4A.2a and Fig 4A.2b).

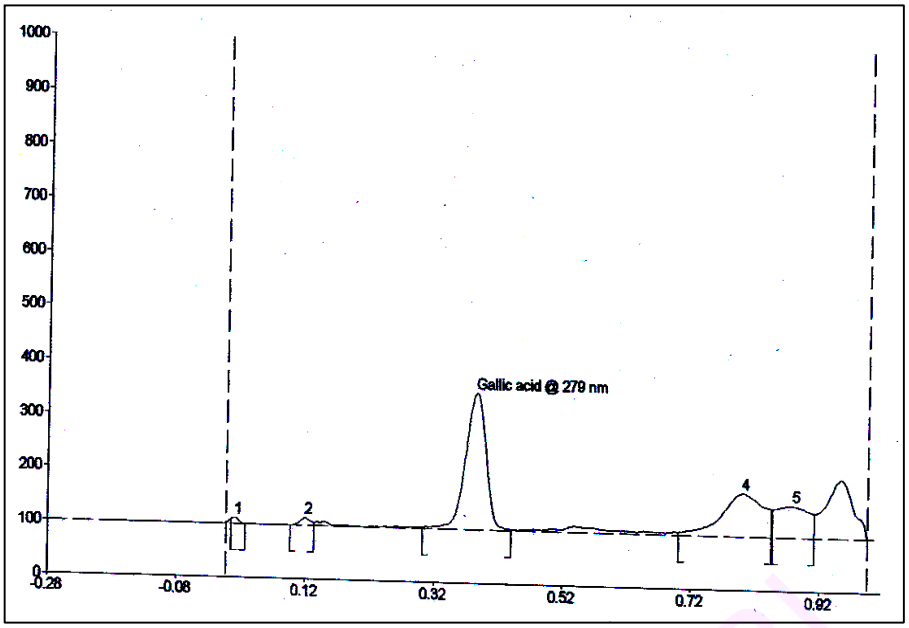
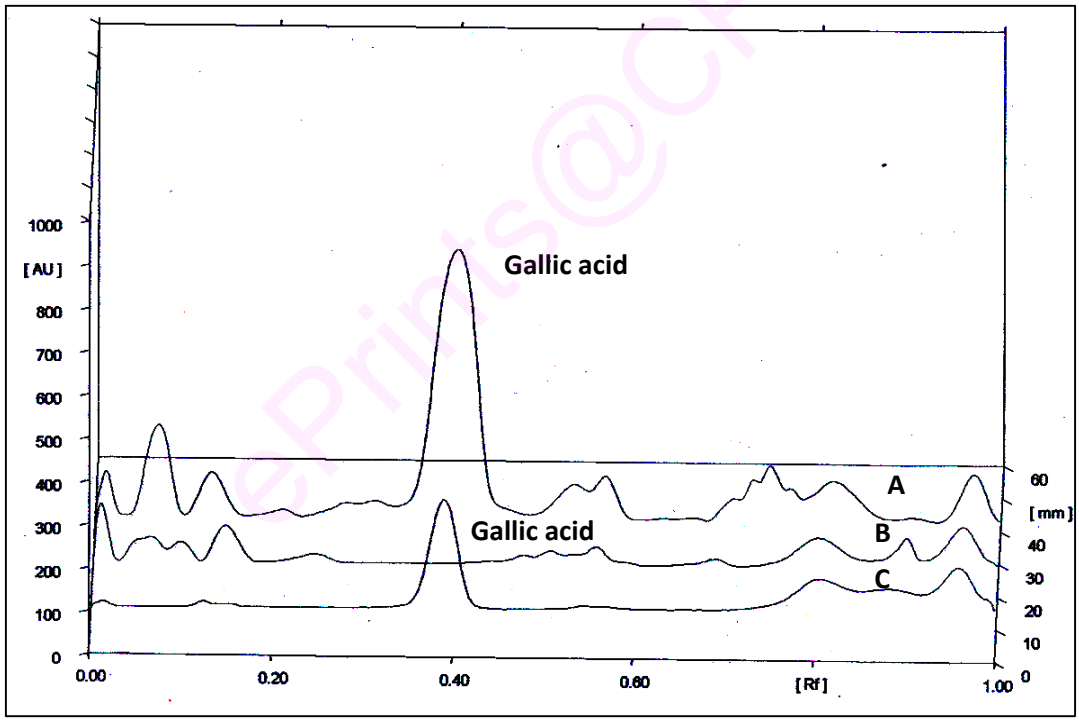


Fig 4A. 1a: HPTLC profile of standard gallic acid that is detectable at 279 nm. The mobile system used was toluene: ethylacetate:formic acid in the ratio; 4:5:1.



4A.1b: HPTL profile water soluble polyphenols of aqueous leaf extract *C. crista* and *C.asiatica*. The graph indicates the presence of gallic acid in aqueous extracts of *C. crista* and absent in *C.asiatica*.. The mobile system used was toluene: ethyl acetate: formic acid in the ratio; 4:5:1. Legend: Track A: Standard Gallic acid, Track B: *C.crista*. Track C: *C.asiatica*

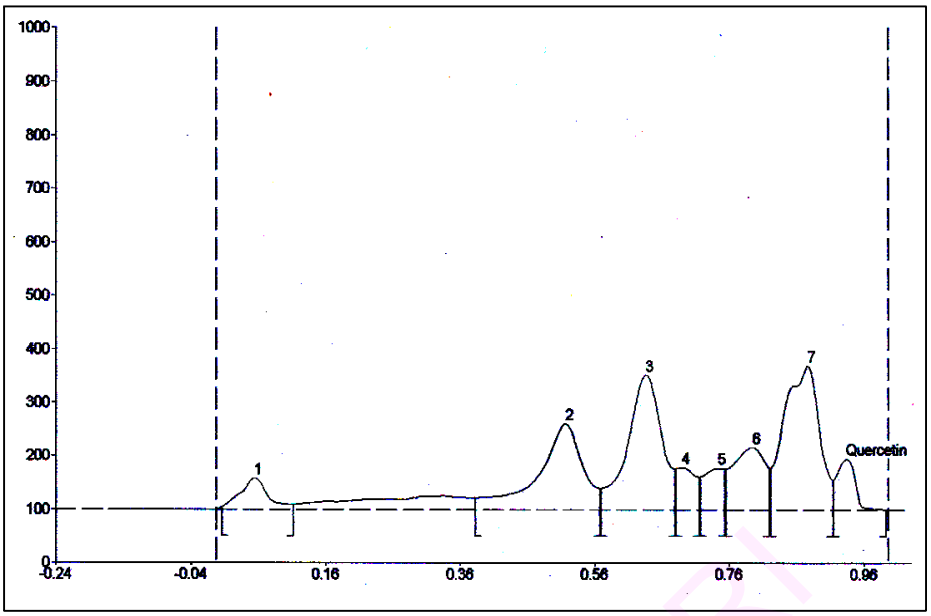


Fig 4A.2a: HPTLC profile of standard quercetin. The solvent system used is formic acid: glacial acetic acid: water in the ratio: 100:11:11:26.

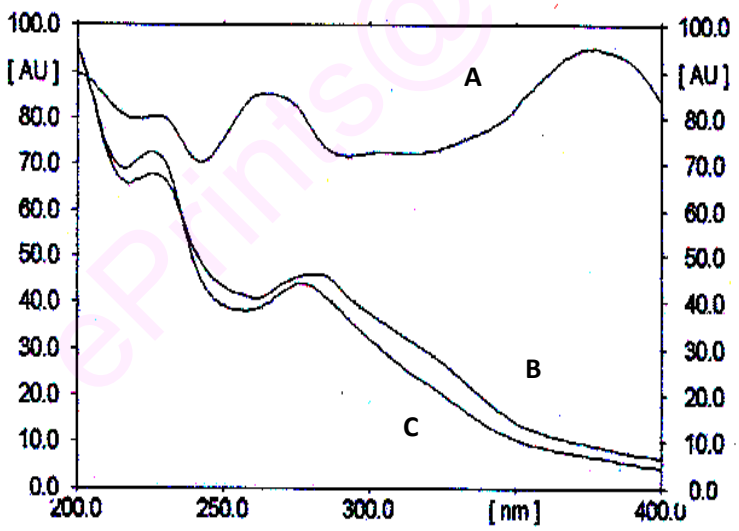


Fig 4A.2b: HPTLC profile of *C. crista* and *C. asiatica* aqueous leaf extract. There were two peaks for quercetin at 378nm and 200nm. Quercetin was present in both the *C. crista* and *C.asiatica*. Legend: Track A: Standard Quercetin, Track B: *C.crista.* Track C: *C.asiatica*

4A. 3.4. Anti-oxidant assays
(i) DPPH Assay

The DPPH scavenging potential of *C. crista*, *C. asiatica* and ascorbic acid was analyzed. C. *crista* and *C. asiatica* extract scavenged the DPPH radical with higher IC50. The ascorbic acid significantly scavenged the DPPH radical compared to *C. crista* and *C. asiatica*. The IC_{50} value of *C. crista*, *C. asiatica* and Ascorbic acids was 24.35± 1.1, 139. 5±2.01 and 14.75±0.72µg/ mL respectively (Table 4A. 5).

Table 4A. 5: DPPH scavenging assay for aqueous extracts of *C. crista* and *C.asiatica*. The inhibition by these two extracts was compared with standard ascorbic acid.

Sl No	**Cc (µg/mL)**	**% Scavenging**	**IC $_{50}$µg/ mL)**
1	5	13.55±1.40	
2	10	14.64±1.81	
3	20	49.27±1.72	
4	30	54.24±1.48	
5	40	65.27±0.78	
6	50	77.53±0.57	
7	100	88.35±0.29	**24.35± 1.1**
8	250	88.77±0.32	
9	500	89.21±0.21	

SlNo.	**C a(µg/Ml)**	**% Scavenging**	**IC $_{50}$µg/ mL)**
1	5	0.32±0.09	
2	10	0.65±0.19	
3	20	5.12±0.75	
4	30	9.17±1.87	
5	40	14.55±1.38	
6	50	19.13±1.55	
7	100	40.74±1.6	**139. 5±2.01**
8	250	86.30±1.56	
9	500	91.56±1.19	

SlNo.	Asc(µg/mL)	% Scavenging	IC $_{50}$µg/ mL)
1	5	29.32±0.46	**14.75±0.72**
2	10	46.14±0.14	
3	20	88.46±0.27	
4	30	94.03±0.71	
5	40	94.17±0.77	
6	50	94.88±0.64	
7	100	96.63±0.4	
8	250	96.7±0.22	
9	500	98.36±0.37	

(ii) Superoxide anion (O2•−) scavenging of *C. crista* and *C. asiatica*

Superoxide scavenging activity assay was done by following the reduction of NBT [Fontana *et al* 2001]. In this assay PMS-NADH coupling results in superoxide radicals. These superoxide radicals have capacity to reduce NBT. The effect of *C. crista* and *C. asiatica* on scavenging of superoxide anions was demonstrated. The ascorbic acid was used as a standard. The aqueous extracts of *C. crista* and *C. asiatica* dose dependently scavenged the superoxide anions. The IC50 values were 280±3.2, and 275±2.9 µg/ mL for *C. crista* and *C. asiatica* respectively. The IC_{50} of standard ascorbic acid is 62.1±1.2µg/ mL (Table 4A. 6).

Table 4A.6: Superoxide scavenging activity of *C. crista* and *C. asiatica* was compared with standard ascorbic acid.

Cc (µg/mL)	**% Scavenging**	**IC($_{50}$µg/ mL)**
50	14.74±1.58	
100	30.38± 5.12	
250	48.79±1.48	**280±3.2**
500	71.69±1.15	

C a(µg/mL)	**% Scavenging**	**IC $_{50}$µg/ mL)**
100	3.47±0.44	
250	49.82±0.63	
500	67.64±1.37	**275±2.9**
1000	69.19±1.29	

Asc (µg/mL)	**% Scavenging**	**IC_{50} (µg/ mL)**
10	20.45±0.42	
50	45.02±0.92	
100	78.10±1.01	
150	90.63±0.34	**62.1±1.2**
250	94.72±0.22	
500	98.52±0.53	

(iii) Reducing potential *C. crista* and *C. asiatica*

The reducing potential of compound/s indicates its ability to act as antioxidant. The reducing power of aqueous extracts of *C. crista* and *C. asiatica* were assessed. The ascorbic acid was used as a standard. A dose dependent increase in reducing power of *C. crista* and *C. asiatica* are observed. *C. crista* was found to be more efficient in reducing ferric to ferrous form of iron compared to *C. asiatica*. However, the standard ascorbic acid showed relatively higher reducing power compared to *C. crista* and *C. asiatica* (Fig 4A.3).

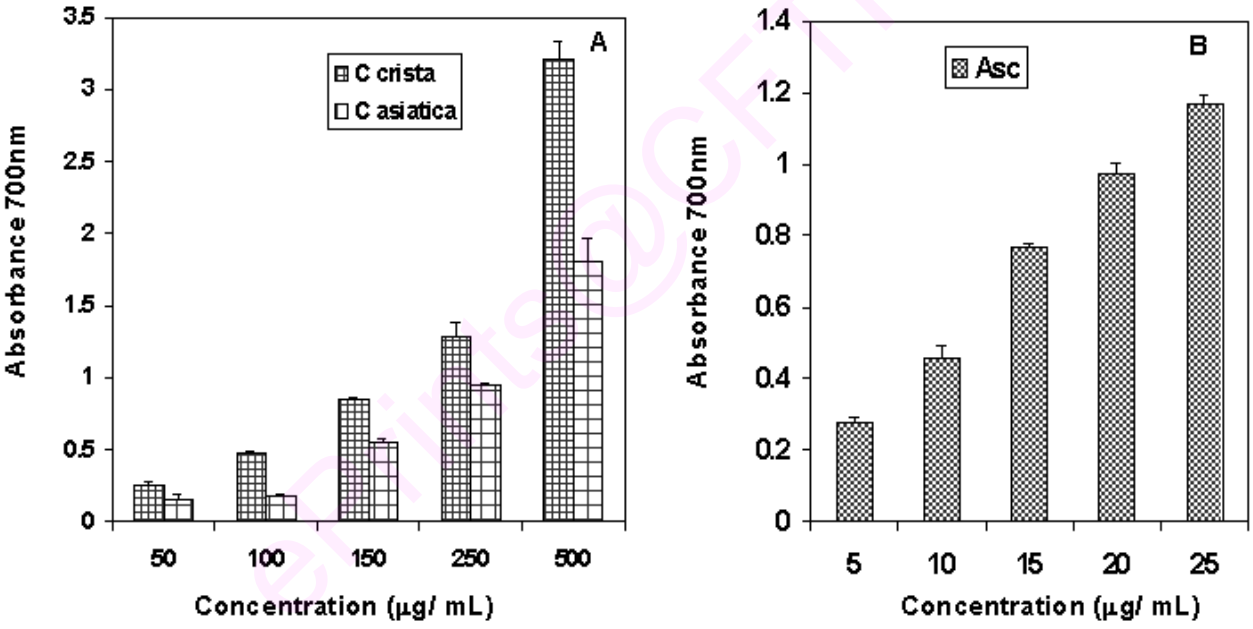


Fig 4A.3: Reducing potential of aqueous extracts of *C. crista* and *C. asiatica.* The reducing potential of the two extracts were compared the standard ascorbic acid.

4A. 3. 5. Anti-inflammatory assay: 5-Lipoxygenase assay

The anti-inflammatory activities of aqueous extracts of *C. crista* and *C. asiatica* was studied using 5-Lipoxygenase assay. The results showed that *C. crista* has showed significant inhibition of 5-Lipoxygenase with IC_{50} of 23 ±1.1µg/ mL compared to *C.asiatica* with IC_{50} of 250 ±2 .81µg/ mL. The standard NDGA has lowest IC_{50} of

8.6± 0.52 µg/ mL (Table 4A. 7). These results indicate that *C. crista* is effective as anti-inflammatory molecule compared to *C.asiatica*

Table 4A 7: 5-lipoxygenase-inhibition activity of *C. crista* and *C. asiatica.* The IC_{50} of two extracts were compared with standard NDGA.

Sl.No.	Extract/ Standard	IC_{50} µg/ mL)
1	*Caesalpinia crista*	**23±1.1**
2	*Centella asiatica*	**250±2.8**
3	NDGA	**8.6± 0.52**

A. 4. Discussion

The process of neurodegeneration is mediated by oxidative stress and inflammation reactions [Patten *et al*., 2010]. The defense system against the oxidative stress and inflammation subsides as ageing progresses [Uttara *et al*., 2009; Sloane *et al*., 1999; Tan and Seshadri, 2010]. The regulation of balance between oxidative stress and the antioxidant defense system play an important role in preventing the deleterious effects of oxidative stress (Smith *et al*., 1997). To enhance this defense system, there is a need to find the effective modulator of both oxidative stress and inflammation molecule. The natural product source is presumed to be safe to human beings with less or no side effects [Anekonda and Reddy, 2005]. There are reports that natural products exhibits antioxidant activities [Ljbunicc *et al* 2006; Liao *et al* 2008; Zhao, 2009; Khan *et al*., 2010]. There are reports on the plant extracts showing both antioxidant and inflammatory activities [Akula and Odhav, 2008, Kim *et al*., 2010; Cowley *et al*., 2010; Ock *et al*., 2010; Ilavarasan *et al*., 2005; Sheeja *et al*., 2006; Jun *et al*., 2005; Gill *et al*., 2010; Santosh and Sohan, 2008; Srivastava *et al*., 2010]. Therefore, there is a great need to screen natural product having both antioxidant and anti-inflammatory activities.

We analyzed aqueous extracts of *C.crista* and *C. asiatica* for phytochemical contents by qualitative assays. We found that these extracts answered positively for qualitative tests for carbohydrates, proteins, aminoacids, phenolics, saponins and flavonoids.

However, these extracts did not show positive results for alkaloids. This will indicate broad range of constituents of the crude extracts and this help in further characterizing the extract. We also subjected the aqueous extracts of *C.crista* and *C. asiatica* for analyzing for its trace metals contents. We evidenced that the aqueous leaf extracts have higher iron, zinc and copper in *C. crista* compared to *C.asiatica*. However, the whole leaf of *C. asiatica* contains more iron, copper and zinc compared to *C. crista*.Additionally, aqueous extracts of *C.crista* and *C. asiatica* are subjected for mapping the chemical composition by HPTLC. The results showed that gallic acid is present in *C. crista* and absent in *C. asiatica* while quercetin is present in both *C. crista* and *C.asiatica*.

Our present study results revealed both the extracts have antioxidant and anti-inflammatory activities. The DPPH scavenging assay is used to evaluate the free radical scavenging potential of various plant extracts [Lee *et al* 2003; Nagai *et al* 2003]. It is a rapid and easy assay to evaluate the both polar and non-polar plant extracts. Our results showed that aqueous leaf extracts of *C. crista* and *C. asiatica* dose dependently showed DPPH radical scavenging activity. Both the extracts showed moderately less DPPH scavenging compared to ascorbic acid, which is used as a standard. Our results are in agreement with other studies [Katbamna *et al* 2008; Mandal *et al* 2009]. The effect of antioxidants on DPPH may be due to their hydrogen donating property. But Foti *et al*. [2004] suggested that an antioxidant acts like an electron transfer. The DPPH radical scavenging ability of the aqueous extracts may be attributed to the water soluble polyphenols present in them.

The superoxide anion is the reduced form of molecular oxygen and plays significant role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical and singlet oxygen [Lee *et al*., 2004]. Superoxide has been found to involve in lipid peroxidation [Wickens, 2001]. Additionally, superoxide is reported to be involved in ischemia [Radi *et al*., 1991]. The aqueous leaf extracts of *C. crista* and *C. asiatica* dose dependently increased the superoxide scavenging. However, aqueous extracts of *C. asiatica* showed comparable superoxide scavenging

activity to *C. crista.* While the standard ascorbic acid showed higher scavenging activity compared to both the extracts.

The iron reducing potential of a compound indicates its ability to act as antioxidant. In potassium ferricyanide reducing assay, the reductants in the extracts may reduce ferric to ferrous by donating electron. The amount of ferrous can be monitored by measuring the complex formation with Perl's Prussian blue at 700nm [Chung *et al* ., 2002]. Our results showed that *C. crista* has more reducing ability than *C .asiatica.* While standard ascorbic acid showed significantly higher reducing potential compared to *C. crista* and *C. asiatica* extracts. We hypothesize that reducing potential of extracts of *C. crista* and *C. asiatica* may be due to the electron donating property of the water-soluble polyphenols present in them [Peñarrieta *et al*., 2008]

The 5-lipoxygense enzymes is a key enzyme in the biosynthesis of leukotriens. 5-lipoxygenase contains non-heme iron in catalytic site. It catalyzes incorporation of dioxygen into unsaturated fatty acid. It mainly converts AA to biologically active leukotriens. These leukotriens are implicated in inflammatory and allergic reactions. The harmful effects of can be prevented by inhibiting its production. So, it can be done by inhibiting the 5-lipoxygenase which catalyses its production. There are number of reports on the natural products being used as antioxidant [Reddy and Lokesh, 1992; Naidu, 1995; Madsen *et al*., 1996; Shobana *et al*., 2000; Zhao, 2009; Khan *et al*., 2010], anti-inflammatory, [Muralidhara and Narasimhamurthy, 1988; Kim *et al*., 2010; Cowley *et al*., 2010; Ock *et al*., 2010; Ilavarasan *et al*., 2005; Sheeja *et al*., 2006; Gill *et al*., 2010; Srivastava *et al*., 2010] and anti-carcinogenic activities [Chen *et al*., 1999; Joe *et a*l., 1997; Kintzios and Spiridon, 2006; Pandey and Madhuri, 2009; Sa and Das, 2008]. There are limited studies on the role of natural products being used as inhibitors of 5-lipoxygenase [Altman *et al*., 2000]. Conversely there are number of inhibitors of 5-lipoxygenase which also act as antioxidants and also inhibit lipid peroxidation [Thody *et al*., 1987; Slater *et al*., 1987]. Our results demonstrated that *C. crista* significantly inhibited 5-lipoxygenase compared to *C. asiatica*. However, these two extracts have lower IC_{50} values compared to standard NDGA. These effects may be attributed to the water- soluble polyphenols present in the extracts. Our results are in agreement with other studies where phenols/ flavonoid

compounds in vegetable/ fruits are shown to modulate activities of 5-lipoxygenase and prostaglandin- H synthase pathways of AA [Laughton *et al*., 1991; Hsuanyu *et al*., 1992; Alanko *et al*., 1993; Alanko *et al*., 1999; Langlois *et al*., 2006].

The brain is rich in polyunsaturated fatty acids; transition metals and ascorbate which make the brain susceptible for oxidative stress bi pro-oxidants [Pratico, 2008]. This results in the susceptibility of the brain to oxidative damage. The oxidative damage in AD is evidenced by injury caused by reactive oxygen species and reactive nitrogen species [Pratico, 2008]. The response of the oxidative stress in AD includes the appearance of amyloid beta in the form of senile plaques and tau as neurofibrillary tangles [Petersen *et al*., 2007]. The markers of oxidative stress in AD includes modified DNA and RNA, protein, and lipids; (i) DNA: 8–hydroxy2-deoxy guanosine, 8-hydroxyguanosine, 8- hydroguanine and DNA breakage, DNA fragmentation and DNA nicking, (ii) protein: protein carbonyls and nitrotyrosine and advanced glycation end products and (iii) Lipids: 4-hydroxynonenal, thiobarbuturic acid reactive substrates, malonaldialdehyde, acrolein, isoprostanes and neuroprostanes. The main source of oxidative damage in AD is the presence of transition metals, abnormal mitochondria and amyloidbeta [Reddy and Beal, 2008]. The higher levels of copper, iron and zinc found in AD brain are responsible for increased generation of reactive oxygen species [Good *et al*., 1992; Lovell *et al*., 1998]. The beta-amyloid also contributes free radicals by producing hydrogen peroxide, which in the presence of iron and copper forms hydroxyl radicals via Fenton reaction. Further, beta-amyloid is reported to induce mitochondrial dysfunction and disrupt the mitochondrial electron transport chain [Reddy and Beal, 2008]. The abnormal mitochondria leak free radicals and cause irrepairable cellular damage in brain (Reddy and Beal, 2008]. The free radicals which are generated in cell induce both structural and functional alterations leading to cell injury ultimately neuronal dysfunction. Further, oxidative stress known to modify the activities of beta- and gamma-secretase to favor the production of higher levels of beta-amyloid [Pratico, 2008].

Antioxidants and antioxidant enzymes have shown to protect against the beta-amyloid induced neurotoxicity. For example, antioxidants are reported to increase the activities of catalase and superoxide dismutase [Behl *et al*., 1994; Qin *et al*., 2002].

Their studies suggest the intake of antioxidants in diet may reduce the risk of developing AD [Gonalez-Gross *et al*., 2001; McDaniel *et al*., 2003].

The oxidative stress and inflammation significantly may contribute to the both initiation and progression of AD [Heneka and Banion, 2007]. The inflammatory response in AD includes the activated microglia and astrocytes in area of beta-amyloid deposits [Heneka and Basion, 2007; Kamer, 2010]. The amyloid deposits activate the complement system called the complement factor C1 which in turn activates the inflammatory response in the form of microglia [Rogers *et al*., 1992; Heneka and Basion, 2007]. Beta-amyloid directly binds to advanced glycation end products on microglia and activates microglia mediated reactions (Baum and Ng, 2004]. The natural product curcumin not only chelate metals and free radicals but also modulates the antioxidant activities such as heme oxygenase [HO-1] [Motterlini *et al*., 2000], glutathione S-transferase [Nishinaka *et al*., 2007] which elevates the glutathione in the brain [Ishrat *et al*., 2009]. The curcumin tested on transgenic mouse over expressing the beta- amyloid showed reduction in the levels of oxidatively modified proteins [Lim *et al*., 2001]. Interestingly, curcumin also prevented the activities of lipoxygenase and cyclo-oxygenase which is involved the synthesis of pro-inflammatory molecules such as leukotriens, thromboxanes and prostaglandins [Nanji *et al*., 2003; Bengmark *et al*., 2006; Sandur *et al*., 2006; Rao, 2007]. Curcumin also known to disrupt the beta-amyloid induced cytokines and chemokines in peripheral blood monocytes and also decreases the levels of interleukin -1β [Giri *et al*., 2004; Lim *et al*., 2001].

The wine consumption and the incidence of AD has been widely debated [Orogozo *et al*., 1997; Lindsay *et al*., 2002]. The lesser incidence of AD in the population who consumes wine is attributed to resveratrol present in the wine [Lindsay *et al*., 2002]. Resveratrol reportedly scavenged the free radicals and protect neurons [Jung and Surh, 2003; Savakasan *et al*., 2003]. The pheochromocytoma -12 cells when incubated with resveratrol known to abolish deleterious effects of the reactive oxygen species [Jang and Surh, 2003]. Additionally, resveratrol also known to increase the antioxidant gluthathione and antioxidant enzymes to combate oxidative stress [Cao and Hi, 2004]. The resveratrol has shown to be neuroprotective effects against streptozotocin induced oxidative stress [Sharma and Gupta, 2002].

Additionally, resveratrol decreased the malondehyde levels in oxidative induced rats [Sharma and Gupta, 2002; Kumar *et al*., 2007]. The *C. asiatica* extract showed improvement in cognitive behavior and prevented oxidative stress in streptozotocin treated rats [Kumar and Gupta, 2003]. The resveratrol shown to act as anti-inflammatory molecule by inhibiting the activation of astrocytes and microglia [Wang *et al*., 2002; Bi *et al*., 2005; Candelario-Jalil *et al*., 2007]. Additionally, resveratrol shown to inhibit the induction of cyclooxygense-2 expression and inducible nitric oxide synthase [Rahman *et al*., 2006]. These studies indicate that resveratrol act as antioxidant and anti-inflammatory role. Further, it also known to enhance the beta-amyloid clearance by inducing the resveratrol [Murambaud *et al*., 2005].

Green tea has attracted attention of researches across for its beneficial health effects. The beneficial effects of green tea are attributed to catechins present in it. The major catechin present in the green tea is EGCG. The EGCG has diverse function that includes activating different signaling pathways, activating antioxidant enzymes, inducing neuroprotective genes etc [Rezai-Zadeh *et al*., 2005; Rezai-Zadeh *et al*., 2008; Singh *et al*., 2008]. The EGCG is known to decrease the production of beta-amyloid by shifting amyloidogenic pathway to non-amyloidogenic pathway by inducing alpha-secretase [Rezai- Zadeh *et al*., 2005]. Additionally, EGCG also chelates metals such as iron and copper and there by prevents the formation free radicals and consequent oxidative damage [Singh *et al*., 2008]. The EGCG also beta-amyloid induced oxidative stress and lipid peroxidation in rat brain [Haque *et al*., 2008; Choi *et al*., 2001]. The anti-inflammatory role of EGCG was confirmed by their inhibitory role on interleukin-6 and interleukin -8 induction [Kim *et al*., 2007]. Also, EGCG suppressed the activation of cytokines, interleukin-1 and beta-amyloid induced cyclo-oxygenase -2 and prostaglandin E2 [Kim *et al*., 2007]. It is also known to prevent the lipo-polysacharide induced microglial activation and there by prevents the inflammation and inflammation mediated neuronal dysfunction [Li *et al*., 2004]. Garlic extract was found to have anti-amyloidogenic property [Gupta and Rao, 2007; Gupta *et al* ., 2008]. So, many of the molecules described in the previous paragraph have anti-amyloidogenic, antioxidant and anti-inflammatory property. In the context of the AD progression which process should be targeted is the daunting question?. The amyloid hypothesis suggests that amyloid precedes the oxidative stress. But

oxidative stress hypothesis opines that oxidative stress precedes the beta-amyloid accumulation. There are many studies showing the plant based molecules reduce the amyloid production [Dhanasekaran *et al*., 2009; Rezai-Zadeh *et al*., 2005]. Also, reports are available on anti-amyloidogenic effects of plant extracts where they have shown the inhibition of amyloid aggregation and also dis-aggregate the pre-formed fibrils [Fujiwara *et al*., 2009, Fujiwara *et al*., 2006; Gupta *et al*., 2007; Gupta *et al*., 2009; Ramesh *et al*., 2010]. These are aimed to reduce the amyloid aggregation and its effects on neuronal dysfunction. Other way of reducing the deleterious effects of amyloid is by clearing the excess amyloid in AD brain. Marambaud *et al*. [2005] has reported that resveratrol induce proteomes to degrade the amyloid protein. So, reducing the amyloid load also reduces the amyloid induced oxidative stress. More emphasis on search for anti-inflammatory molecules is necessary to combat both inflammation and anti-AD effects. The aqueous extract of *C. crista* has shown anti-amyloidogenic, antioxidant and anti-inflammatory activities where as *C. asiatica* has shown anti-oxidant and anti-inflammatory activities. So, the *C. crista* has shown all the potential to possibly become the drug candidate for AD. The *C. crista* and *C.asiatica* leaf extract was further used to evaluate for its anti-amyloidogenic property which is given in the Chapter 4B and 4C respectively.

Chapter 4B

Anti-amyloidogenic property of C. crista

Chapter 4B Anti-amyloidogenic property of *C. crista*

4B.1. Introduction

Alzheimer's disease is characterized by loss of memory, cognitive dysfunction and alterations in behavior [Selkoe, 2001]. The hallmark pathology of AD has been the deposition of Aβ in the form of senile plaques [Selkoe, 2001]. Aβ can self-assemble to form dimers, soluble oligomers, and protofibrils and diffuse plaques through multistep–nucleated polymerization [Lambert *et al*., 1998]. The evaluation of *in vitro* Aβ self-assembling will provide an opportunity to screen molecules for anti-amyloidogenic property. The prevention of the formation of oligomers and the fibrils from soluble monomers is of therapeutic significance for AD drug discovery [Smith *et al*., 2007]. There are reports on the crude extracts or pure compounds from plants having anti-amyloidogenic properties [Fujiwara *et al*., 2009; Gupta and Rao, 2007; Gupta *et al*., 2009; Lau *et al*., 2007; Papandreou *et al*., 2006], but the mechanisms of their therapeutic potential are still not clear.

The present study aims to understand the inhibitory effects of leaf aqueous extract of *C crista* on Aβ(1-42) aggregation. *C. crista* Linn (Syn C. bonducella [L.] Roxb.) belongs to family Fabaceae, found abundantly in tropical and subtropical regions of Southeast Asia. It is found in the hot and humid regions of India, Myanmar and Sri Lanka. In India, it is known as Gugugu, Gaduggu, Katukaranja and it is widely distributed in Karnataka, West Bengal, Kerala and Maharashtra states of India. The seed kernel of *C. crista* contains Cassane- and norcassane-type diterpenoids [Kalauni *et al*., 2005]. The stem and roots also known to contain new type of diterpenes [Cheenpracha *et al*., 2005]. The seeds are traditionally used for anthelimintic, antimalarial, antipyretic and anti-inflammatory properties. The tribal knowledge base from India insighted that aqueous extract of *C. crista* is used as mental stress relaxation health drink by forest dwellers. There are limited studies on anti-diuretic, antibacterial, anti-diabetic and antioxidant potentials of *C. crista* [Dhar *et al*., 1968; Kalauni *et al*., 2005; Katbamna *et al*., 2008; Neogi and Nayak, 1958; Rao *et al*., 1994]. In the present study, an investigation was carried out on anti-amyloidogenic effects of *C. crista* aqueous extract by using *in vitro* model as an early quick battery test before going to transgenic animal studies. We have used three methods to

understand the effect of *C. crista* in preventing the formation of; i) aggregates from monomers (ii) aggregates from oligomers and also (iii) dis-aggregation of preformed fibrils of Aβ.

4B.2. Materials and Methods

Aβ(1-42) was purchased from EZ Biolabs, USA. Tris buffer, glycine, sodium hydroxide, hydrochloric acid were from SRL, India. Thioflavin–T was purchased from ICN Biomedicals Pvt. Ltd, USA. Copper grids (200 mesh size) were purchased from Sigma chemicals, USA. Uranyl acetate was purchased from BDH Laboratory chemicals Division, India.

C. crista was obtained from Western Ghats of Karnataka, India and the species of the plant was identified by Botanist (Taxonomic deposit number: 417358). The aqueous extract of leaves was prepared. 40 g of dried leaf was washed in triple distilled water. The washed leaf was boiled in steam extractor (2 litre of triple distilled water for ~3 hr till the water content become half). The extract was filtered through Whatman 42 to get the clear solution and lyophilized to powder [Yield – 2.5% w/w].

4B.2.1. Estimation of total polyphenols in leaf extract: Estimation of total polyphenols in leaf aqueous extract: The lyophilized aqueous extract was analyzed for total polyphenols [Swain and Hills, 1959]. Briefly, 10µl of aqueous extract (100 mg / ml) was taken and added with 490µl of milli Q water and 500 µl of FC reagent (Folin-Ciocalteu reagent 1:2) and incubated at room temperature for 3 min. After 3 min, 1ml of saturated sodium carbonate was added and the reaction mixture was incubated at room temperature for 60 min. The absorbance was recorded at 675nm. The gallic acid was used as a standard and from the standard graph, the concentration of total polyphenols was calculated.

4B.2.2. Evaluation of anti-amyloidogenic property of *C. crista*

The Aβ(1-42) was dissolved in triple distilled water and centrifuged at 10,000 rpm for 10 min and supernatant was taken for monomers and aggregates if any were precipitated. Experiments were designed to evaluate the anti-amyloidogenic property of *C. crista* using three phase study protocol. Phase I: To understand the prevention of Aβ(1-42) aggregates from monomers. Freshly prepared Aβ(1-42) monomers were co-incubated with *C. crista* aqueous extract from 0 h and studied the aggregation

kinetics to follow monomers-oligomers-aggregates phases as a function of time (0-96 h) and aliquots were taken at 0, 6, 20, 72 and 96 h for thioflavin-T and TEM study. Phase II: To understand the prevention of formation of aggregates from oligomers: Freshly prepared Aβ(1-42) monomers were allowed to form oligomers till 20 h and then extract was added and then studied the formation of aggregates from oligomers as function of time (20-96 h) and aliquots were taken at 20, 36, 48, 72 and 96 h for thioflavin-T and TEM study. Phase III: To understand the efficiency of extract to dis-aggregate the pre-formed fibrils: Freshly prepared Aβ(1-42) monomers were allowed to form matured fibrils for 96 h and then extract was added and followed the dis-aggregation of fibrils for 9 days.

Phase I reaction mixture was as follows: 100μM Aβ(1-42) was incubated with 100μg of aqueous extracts of *C. crista* in a total reaction mixture of 300μl in Tris-HCl buffer pH 7.4 at 37^0C at 0 h. Aliquots of 20μl were drawn each time from incubated sample at intervals of 0, 6, 20, 72 and 96 h.

Phase II: Phase II: Aβ(1-42) was incubated for 20 h to form oligomers and the extract was added at 20 h and aliquots were taken at 20, 36, 48, 72 and 96 h time periods. The oligomers of Aβ(1-42) was prepared as described by Chromy *et al*. [2003].

Phase III: Aβ(1-42) was incubated for 96 h to form matured fibrils and then incubated with the extract for 9 days and aliquots were drawn.

4B.2.3.Thioflavin T assay: Thioflavin-T assay was conducted to study Aβ aggregation kinetics. Thioflavin-T specifically binds to aggregates. 25μl of (1mM) thioflavin-T was added to 1000μl of total reaction volume. Thioflavin-T fluorescence was measured at an excitation and emission wavelengths of 446nm and 482nm, respectively using a F4500 Hitachi fluorescence spectrometer. The background thioflavin-T fluorescence intensity was subtracted from the experimental values.

4B.2.4.Transmission Electron Microscopy study: Transmission Electron microscopy (TEM) study was conducted to detect presence or absence of aggregates. 10μL of incubated sample was placed on carbon coated copper grid (200 mesh size) and allowed for one minute and excess sample was wicked off with lens paper and

then negatively stained by transferring the grid face down to a droplet of uranyl acetate (2% w/v) for one min before wicking off the solution. Then the grids were air dried for an hour. Four individual experiments were carried out for each sample. The grids were completely dried to avoid moisture and then scanned under JOEL 1010 TEM. All the data is statically analyzed using Microsoft Excel-2003.The concentration of polyphenols was found to be 80 mg / gm (0.8% w/w) of aqueous extract.

4B.3.Results

4B.3.1. Phase I: *C. crista* inhibits Aβ(1-42) aggregate formation from monomers:

(i) Thioflavin T- study: Fig 4B. 1A shows three phases of aggregation kinetics of Aβ(1-42) monitored through thioflavin-T fluorescence as a function of time (0-96 h). The aggregation kinetics followed a sigmoidal curve. The thioflavin –T data indicated a lag period upto 20 h, where thioflavin-T fluorescence intensity is static indicating the presence of monomers only. After 20 h, there is an intermediate phase from where oligomers and other intermediate forms might have formed till 48 h. The other one is the saturated phase where, fully matured fibrils are formed, where thioflavin-T fluorescence is static. This phase is from 48 to 96 h. In the presence of the aqueous extract, the sigmoidal pattern of Aβ(1-42) aggregation kinetics has been prevented (Fig 1A). Hence there was only lag phase with possibly monomers only. Fig 4B.1B shows the thioflavin-T fluorescence at 0, 6, 20, 72 and 96 h time intervals. For Aβ(1-42) alone, as the time increases, the thioflavin-T fluorescence increases and in the presence of aqueous extract, thioflavin-T fluorescence did not increase, indicating that extract inhibited the aggregation. The samples represented in Fig 1B are taken for TEM study.

(ii) Electron microscopic study:

Fig 4B. 1C shows aggregation assay using TEM at different time intervals (0, 6, 20,72 and 96 h). The TEM data clearly shows that when Aβ(1-42) alone was allowed to aggregate from 0 to 96 h, there is clear time dependent aggregation kinetics. There are no aggregates from 0 to 20 h. The aggregates start growing from 72 and 96 h (Fig 4B.1C). But in the presence of *C. crista*, there were no aggregates from 0 to 96 h indicating that *C. crista* totally abolished the aggregation formation (Fig 4B. 1C).

Both Thioflavin-T and TEM data clearly supports that the extract totally prevents the formation of aggregates.

4B.3.2. Phase II: *C. crista* inhibits Aβ(1-42) aggregate formation from oligomers:

(i)Thioflavin –T study: Fig 4B. 2A shows the results of phase II: Thioflavin-T fluorescence data of Aβ(1-42) with and without extract at different time intervals (20, 36, 48, 72 and 96 h). Aβ(1-42) is allowed to aggregate for 20 h, and at 20 h, extract is added and the Aβ(1-42) aggregation process is monitored at different time intervals. The thioflavin-T fluorescence was increased from 20 to 96 h indicating the formation of fibrils from oligomers stage. In the presence of *C. crista*, the thioflavin-T fluorescence values were significantly decreased indicating that the extract has inhibited the formation of fibrils. These results are further confirmed by TEM data as shown below.

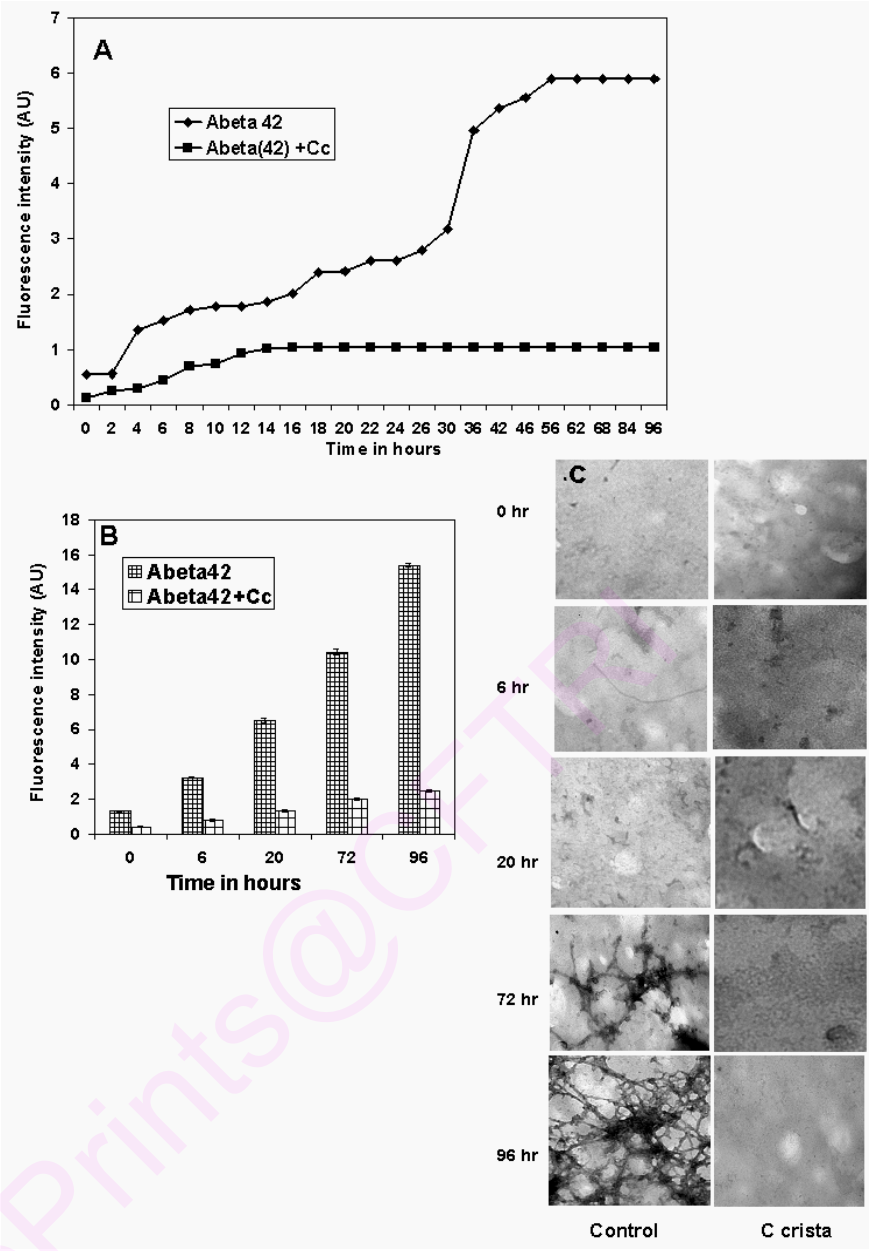


Fig 4B.1A: Effect of *C. crista* on formation of amyloid fibrils from monomers (Phase I): The reaction mixture containing 50μM Aβ(1-42) co-incubated in absence or presence of 100 μg of extract at 37°C in Tris-Cl buffer (pH 7.4). The aliquots were taken from 0 to 96 h at every 2h interval. The fluorescence of thioflavin–T is recorded with excitation and emission wavelength of 446nm and 482nm respectively (Fig 4B.1A). The reaction mixture containing 50μM Aβ(1-42) co-incubated in absence or presence of 100 μg of extract at 37°C in Tris-Cl buffer (pH 7.4). Aliquots are taken at 0, 6, 20, 72 and 96 h. The fluorescence of thioflavin –T is recorded with excitation and emission wavelength of 446nm and 482nm respectively. Fig 4B.1B: Bar diagram showing the thioflavin-T fluorescence of Aβ(1-42) in presence or absence of *C. crista* leaf extract. Fig 4B.1C:Transmission Electron Microscopic study: The reaction mixture contain 50μM Aβ(1-42) co-incubated in absence or presence of 100 μ□g of extract at 37°C in Tris-Cl buffer (pH 7.4). The aliquots are taken at 0, 6, 20, 72 and 96 h and analyzed for presence or absence of aggregates.

(ii) Electron microscopic study: Fig 4B. 2B shows the results of TEM of phase II : sssThe formation of fibrils increased with time from 20 to 96 hrs in Aβ(1-42) alone. But in the presence of *C. crista*, no fibrils were observed in Aβ(1-42) as a function of time (0-96 hrs). This indicates that *C. crista* could able to prevent fibril formation even after the aggregation process was initiated at 20 hrs (Fig 4B. 2B).

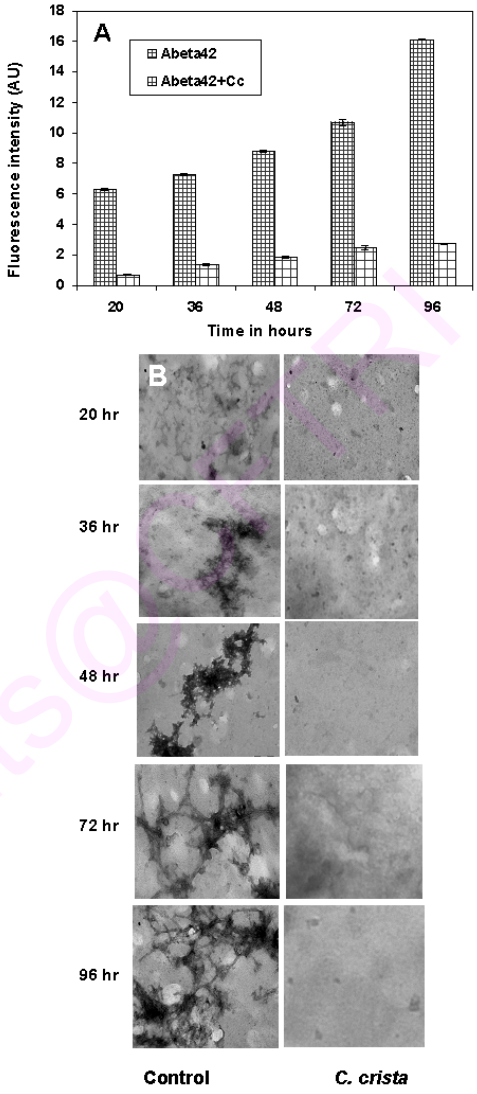


Fig 4B.2A: Inhibition of formation of amyloid fibrils from oligomers by *C. crista* aqueous extract (Phase II): Bar diagram showing the thioflavin-T fluorescence of Aβ(1-42) as a function of time: Aβ(1-42) is allowed to form oligomers for 20 hrs and *C. crista leaf* extract is added at 20 hrs of incubation. Aliquots are taken at 20, 36, 48, 72 and 96 hrs and thioflavin-T fluorescence emission was measured at 482nm. Fig 4B. 2B: Transmission Electron Microscopy study: Aβ(1-42) is incubated for 20 hrs and *C. crista* leaf extract is added at 20 hrs of Aβ(1-42) incubation. Aliquots are taken at 20, 36, 48, 72 and 96 hrs. Aliquots are analyzed for the presence or absence aggregates using electron microscopy.

4B.3.3 Phase III: Phase III: *C. crista* dis-aggregated preformed Aβ(1-42) fibrils: **(i) Thioflavin-T study**: Fig 4B. 3A shows the results of thioflavin-T fluorescence assay of phase III. The fluorescence was increased form 96 to 9 days in Aβ(1-42) alone. But *C crista* extract significantly decreased the thioflavin-T fluorescence indicating dis-integration of matured fibrils both after 96 hrs and 9 days incubation. **(ii) Electron microscopic study**: Fig 4B.3B shows the results of TEM of phase III: At 96 hrs, there were matured fibrils with extensive branching in Aβ(1-42) alone sample. The extract was added to 96 hrs-matured fibrils and then incubated for another 96 hrs and extended to 9 days incubation. The extract has dis-aggregated the pre-formed fibrils both at 96 hrs and 9 days incubation.

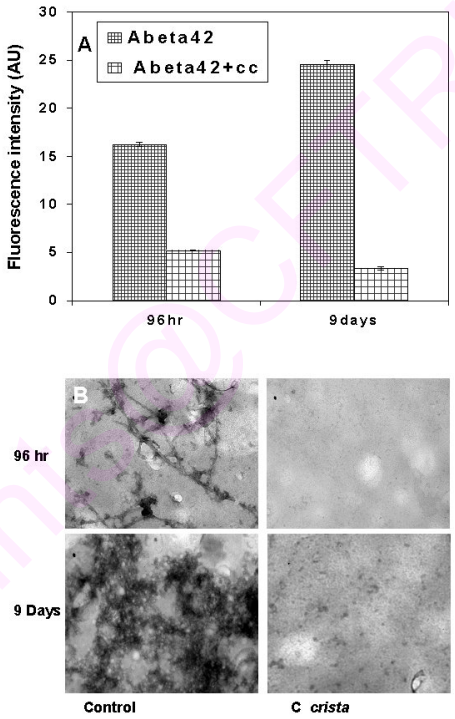


Fig 4B.3A: Dis-aggregation of pre-formed fibrils by *C. crista* leaf extract (Phase III): Bar diagram showing the thioflavin-T fluorescence of Aβ(1-42): Aβ(1-42) is incubated for 96 hrs and *C. crista* leaf extract is added at 96 hrs and Aβ(1-42) is allowed to aggregate further for 96 hrs and extended to 9 days. Thioflavin-T fluorescence measured at emission at 482nm. Fig 4B. 3B: Transmission Electron Microscopy study: Aβ(1-42) is incubated for 96 hrs and then *C. crista* leaf extract is introduced and allowed to aggregate further for 96 hrs and extended to 9 days. The aliquots are analyzed for the presence or absence of aggregates in electron microscopy.

4B.4.Discussion

Alzheimer's disease has a complex pathology with multifactorial mental illness, which is characterized by loss of memory and cognition. The etiological factors include oxidative stress, inflammation, Aβ over expression, elevation in metals etc [Markesbery, 1997; Neve and Robakis, 1998; Roger *et al*., 1996; Selkoe, 2001 Smith *et al*., 1997]. To date, there are no successful drugs for the clinical management of AD. The studies have targeted drug screening for decreasing Aβ levels either through inhibiting the generation of Aβ or reducing levels and enhancing the Aβ clearance from the brain (both by reducing its production and clearance of excessive peptide) [Lau *et al*., 2007; Marambaud *et al* ., 2005; Rezai-Zadeh *et al*.,2005; Rottkamp *et al* ., 2000]. Currently, the studies are focusing to discover natural products as alternate therapeutic molecules for AD management [Fujiwara *et al*., 2009; Fujiwara *et al*., 2006; Joshi and Parle, 2006; Papandreou *et al*., 2006; Porat *et al*., 2006; Wang *et al* ., 2008; Watanabe, 1997].

The natural and synthetic molecules are targeted to reduce the amyloid load both *in vitro* and *in vivo* models [Fujiwara *et al*., 2009; Howes *et al*., 2003; Papandreou *et al* .,2006]. The natural products like *Hypericum perforatum* and *Ginkgo biloba* are found to enhance memory and cognition and possibly reduce the risk for AD [Howes *et al*., 2003]. The studies on *Centella asiatica* extract using PSAPP mouse model of Alzheimer's disease showed that extract decreased the levels of Aβ(40) and Aβ(1-42) in hippocampus but not in the cortical region. Additionally, the reduction in Aβ levels did not correlate Y-maze or open field behaviors in mouse model [Dhanashekaran *et al*., 2009] thus, puzzling the insight *Centella asiatica* as a drug candidate. But the mechanisms underlying the delaying process of disease is not clear. The *Ginkgo biloba* extract has found to enhance the working memory and information processing [Kennedy *et al*., 2000; Solomon *et al*., 2002; Wesnes *et al*., 2000]. The herb *Paeonia suffruticosa* and its active constituents not only inhibited the formation of Aβ fibrils but also de-stabilized the pre-formed fibrils [Fujiwara *et al*., 2009; Fujiwara *et al*., 2006]. The curcumin was reported to inhibit the Aβ fibril formation and also destabilized the pre-formed Aβ fibrils *in vitro* [Ono *et al*., 2004; Yang *et al*., 2005]. The polyphenols like EGCG and resveratrol reported to reduce Aβ production and

also increase Aβ clearance [Marambaud *et al* ., 2005, Rezai-Zadeh *et al*., 2005]. All the above studies showed that natural products are able to reduce amyloid load but could not successfully pass through clinical trials [Marambaud *et al*., 2005; Rezai-Zadeh *et al*., 2005]. Hence, there is still a search operation for better molecules from nature for the management of AD.

The limited studies showed that *C. crista* has anti-oxidant, anti-inflammation and antiviral properties [Dhar *et al*., 1968; Kalauni *et al*., 2005; Luo *et al*., 2002; Mandal *et al*., 2009]. Further, the traditional knowledge also gave an insight that *C. crista* aqueous extract is used as mental relaxation drink by people residing in forest areas. This tempted us to undertake a study to investigate whether aqueous extract of *C. crista* could be able to prevent amyloid aggregation using *in vitro* models. We found that the aqueous extract of *C. crista* could not only prevent Aβ fibril formation but also dis-aggregated Aβ fibrils. The anti-amyloidogenic property of *C. crista* may be attributed to the polyphenols present in the aqueous extract. A study by Ono *et al*. [2004] showed that water-soluble polyphenol; tannic acid inhibited the amyloid fibril formation. However, the mechanism of inhibition of amyloid fibril formation is not clear. In literature, several mechanisms are proposed regarding the role of polyphenols in preventing amyloid aggregation [Burley *et al*., 1985; Gazit, 2002;Gazit, 2002; Pawar *et al*., 2005; Porat *et al*., 2006]. So, for the inhibitory role of polyphenols against fibril formation is attributed to anti-oxidant features of phenolic groups. Porat *et al*. [2006] proposed a new mechanism on polyphenols inhibiting amyloid fibril formation. These efficient polyphenols contain two phenolic rings having 2-6 atom linkers and three OH groups on aromatic ring. These structural features are needed for the non-covalent interactions with beta sheet structures seen in amyloid fibril structures. This may be the main reason to explain why polyphenols effectively inhibit fibril formation from misfolded oligomers. The above structural features do not support the ability of polyphenols interacting with monomers and hence they cannot prevent early nucleation phase. It has been proposed by Porat *et al*. [2006] that amyloidogenic region contains aromatic aminoacids like phenylalanline and tryptophan and favor ordered self assembly [Gazit, 2002; Pawar *et al*., 2005]. The phenol ring present in polyphenol has a novel-stacking mode to interact with aromatic

aminoacid residues. But this feature alone cannot inhibit fibril formation and needs further work in this direction [Taniguchi *et al*., 2005]. We propose that similar mechanism may be involved in preventing amyloid fibril formation and also destabilization of pre-formed fibrils by water-soluble polyphenols from *C. crista*. This data provides a novel indication that *C. crista* may be a potential natural product intervention in AD.

Chapter 4C

Studies to understand the effect of C. asiatica on Aβ(1-42) aggregation in vitro

Chapter 4C Studies to understand the effect of *C. asiatica* on Aβ(1-42) aggregation in vitro

4C.1. Introduction

Amyloids are a group of misfolded proteins that are implicated in the neurodegenerative disorders such as Parkinson's disease, Huntington disease, Frontotemporal dementia and Alzheimer's disease (AD) [Koo *et al*., 1999]. The amyloid β (Aβ) is one such amyloid protein strongly implicated in AD [Selkoe, 2001]. The excessive production and accumulation of Aβ believed to be one of the major risk factor for AD [Tanzi and Bertram, 2005; Tew *et al*., 2008]. Aβ undergoes conformational change and forms deposits in the form of insoluble senile plaques in AD brain [Klein, 2002]. The major therapeutic approaches in AD are towards the reduction of Aβ either by decreasing its production or to enhance clearance of the accumulated Aβ[Demattos *et al*., 2001; Demattos *et al*., 2002; Gelinas *et al*., 2004; Gupta *et al*., 2007; Gupta *et al*., 2009; Morgan *et al*., 2000; Schenk *et al*., 1999]. The accepted concept is that Aβ oligomers are toxic to neurons and induce cell death [Tew *et al*., 2008; Lesne *et al*., 2006; Shankar *et al*., 2008]. The therapeutic approaches in AD include acetylcholineesterase inhibitors, antioxidants, anti-inflammatory and anti-amyloidogenic agents as targets [Giri *et al*., 2004; Dhanashekaran *et al*., 2009; Lim *et al*., 2001; Mukherjee *et al*., 2007; Park *et al*., 2002; Sharma and Gupta, 2002; Smith and Luo, 2004; Wu *et al*., 2002; Yang *et al*., 2005]. The anti-amyloidogenic approach is currently active [Park *et al*., 2002; Yang *et al*., 2005; Feng *et al*., 2009; Fujiwara *et al*., 2009]. There are number of drug targets focused against amyloid load reduction and many of them have not reached the clinical trials [Feng *et al*., 2009; Fujiwara *et al*., 2009; Fujiwara *et al*., 2009; Ono *et al*., 2004; Stackman *et al*., 2003; Wang *et al*., 2008]. Now, the studies have been focused on natural products as alternative candidates for evaluating therapeutic potential against AD [Smith and Luo, 2004; Wu *et al*., 2002; Yang *et al*., 2005; Feng *et al*., 2009; Fujiwara *et al*., 2009; Fujiwara *et al*., 2009; Ono *et al*., 2004]. The Indain penny wort (*Centella asiatica)* has been traditionally used in Asia to cure various ailments. The dried leaves of Indian penny wort is mixed with milk and consume as memory improving [Manyam, 1999; Kirtikar and Basu, 1993] and this is practiced traditionally in selected regions in India

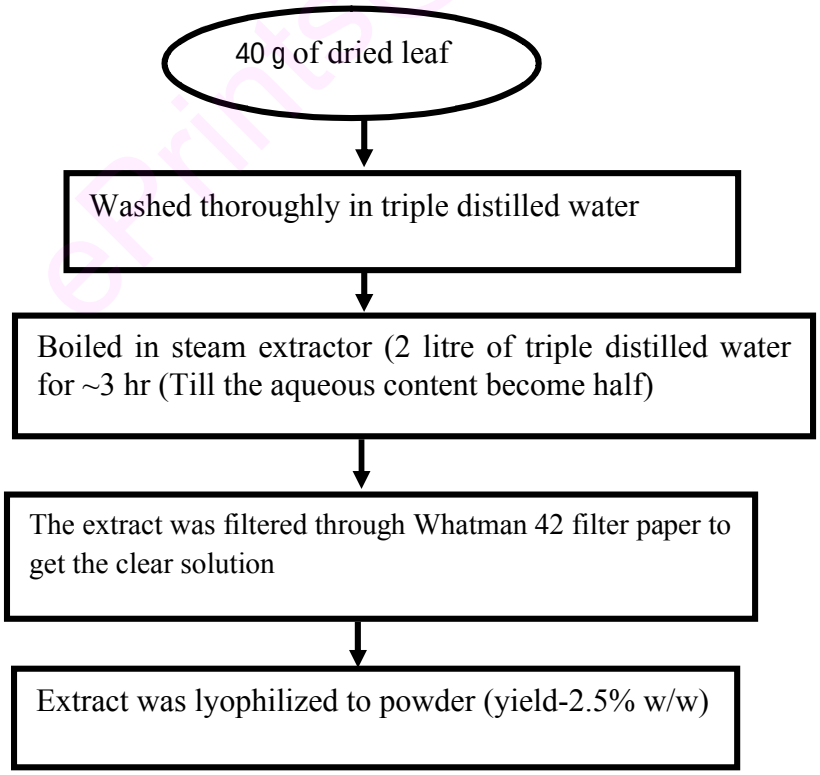
[Nadkarni, 1954]. There are studies on diverse effects of C. *asiatica* such as acetylcholine-esterase inhibition, antioxidant, neuroprotection, and amyloid load reduction [Dhanasekaran *et al*., 2009, Mukherjee *et al*.,2007; Kumar and Gupta, 2002; Gupta *et al*., 2003; Kumar *et al*., 2009]. However, there are no mechanistic studies to understand whether *C. asiatica* prevents Aβ aggregation. Therefore in the present study attempts were made to prevent the aggregation of Aβ (1-42) peptide using C. *asiatica* extract. Aqueous extract of *C. asiatica* as it is traditionally used by local population in western ghats as brain tonic.

4C.2. Materials and methods

Aβ(1-42) was purchased from EZ Biolabs, USA. Tris buffer, glycine, sodium hydroxide, hydrochloric acid were from SRL, India. Thioflavin –T was procured from ICN Biomedicals Pvt. Ltd, USA and Copper grids (200 mesh size) were brought from Sigma chemicals, USA and uranyl acetate was procured from BDH Laboratory chemicals Division, India.

The *C asiatica* was procured from local vegetable market, Mysore and it was identified by an authenticated botanist (Taxonomic deposit number is 9831).

Flow chart for the preparation of aqueous-leaf extract of *C. asiatica*



Dried leaves (40g) of *C. asiatica* was washed thoroughly in triple distilled water for four times. Two litres of triple distilled water was added to steam extractor, boiled till it becomes half the volume (one litre). Aqueous extract thus obtained was filtered using Whatman 42 filter paper to get clear solution. The clear solution was lyophilized to get dry powder. The yield of the extract was 2.5% (w/w).

To evaluate the anti-amyloidogenic property of aqueous extract of *C. asiatica*, the following experiments were designed *in vitro* using the three-phase study protocol. Phase I: To understand the prevention of Aβ aggregation from monomer. Phase I reaction mixture was as follows: 100μM of Aβ was incubated with 100μg of lyophilized aqueous extracts *of C. asiatica* in a total reaction mixture of 300μl containing 10mM Tris-Cl (pH 7.4) at 0 h at 37 ^{0}C. Aliquots of 20μl (10 μM) were drawn each time from incubated sample at intervals of 0, 6, 20, 72 and 96 h.

Phase II: To understand the prevention of formation of aggregates from oligomers. Freshly prepared Aβ was allowed to form oligomers till 20 h following the protocol described by Chromy *et al*.[2005] with slight modification [Chromy *et al*., 2003] and then 100μg of lyophilized extract was added. The aggregation kinetics was studied to follow the formation of aggregates from oligomers as function of time (20-96 h) and aliquots were taken at 20, 36, 48, 72 and 96 h for thioflavin-T and TEM study.

Phase III: To understand the efficacy of extract to dis-integrate the pre-formed fibrils. Freshly prepared Aβ was allowed to form matured fibrils by 96 h and then 100μg of lyophilized extract was added and followed the dis-integration of fibrils at 8 days.

4C.2.1 Thioflavin-T assay:

The thioflavin-T assay was followed to study Aβ aggregation kinetics. Thioflavin-T specifically binds to aggregates but not to soluble monomers. 25μl of (1mM) thioflavin-T was added to 1000μl of total reaction volume containing 5μM Aβ. The thioflavin-T fluorescence was measured at an excitation and emission wavelengths of 446nm and 482nm, respectively using a F4500 Hitachi Fluorescence Spectrometer. The background thioflavin-T fluorescence intensity was subtracted from the

experimental values. The thioflavin-T fluorescence data was analyzed for standard error using origin 6.0.

4C.2.2. Transmission Electron microscopy (TEM) study

Transmission Electron Microscopy (TEM) study was conducted to detect presence or absence of aggregates. Incubated sample (10μL) was placed on carbon coated copper grid (200 mesh size) and allowed for one min and excess sample was wicked off with lens paper and then negatively stained by transferring the grid face down to a droplet of (2% (w/v) uranyl acetate for one min before wicking off the solution. Then the grids were air dried for an hour. Four individual experiments were carried out for each sample. The grids were completely dried to avoid moisture and then scanned under JOEL 1010 TEM.

4C.3. Results

The effect of *C. asiatica* on the Aβaggregation is analyzed as follows:

4C.3. 1. Phase I: Inhibition of the formation of aggregates from monomers: Fig 4C.1A shows three phases of aggregation kinetics of Aβ as monitored by thioflavin-T fluorescence as a function of time (0-96 h). The aggregation kinetics followed a sigmoidal curve. The thioflavin –T data indicated a lag period upto 20 h, where thioflavin-T fluorescence intensity is static indicating the presence of monomers only. After 20 h, there is an intermediate phase from where oligomers and other intermediate forms form till 48 h. The thioflavin–T fluorescence steeply increases in this phase indicating formation of misfolded intermediates. The other phase is the saturated phase where fully matured fibrils are formed. This phase is from 48 to 96 h. The thioflavin –T fluorescence is higher and static in nature indicating matured fibril formation. In the presence of the aqueous extract, the sigmoidal pattern of Aβ aggregation kinetics is followed a similar pattern with no significant reduction in fluorescence intensity. This indicates that *C. asiatica* could not be able to prevent the Aβ aggregation from monomers. Fig 4C. 1B shows the thioflavin-T fluorescence at 0, 6, 20, 72 and 96 h time intervals. For Aβ alone, as the time increases, the thioflavin-T fluorescence increases. And in the presence of aqueous extract, thioflavin-T fluorescence do not significantly altered; supporting the concept that extract could not able to prevent Aβ aggregation. The samples represented in Fig 4C. 1B are taken for TEM study. Fig 4C.1C shows results of TEM study for the presence

or absence of aggregates at different time intervals (0, 6, 20,72 and 96 h). The TEM data clearly shows that when Aβ alone is allowed to aggregate from 0 to 96 h, the formation of fibrils is in time dependent aggregation kinetics. There are no aggregates from 0 to 20 h. The aggregates start growing from 72 and 96 h (Fig 4C.1C). Even in the presence of *C. asiatica*, both at 72 and 96 h the aggregates are seen indicating that the extract could not able to totally prevent the formation of fibrils (Fig 4C. 1C). Thus, thioflavin-T and TEM data clearly supports that the extract could not able to totally prevent the formation of aggregates.

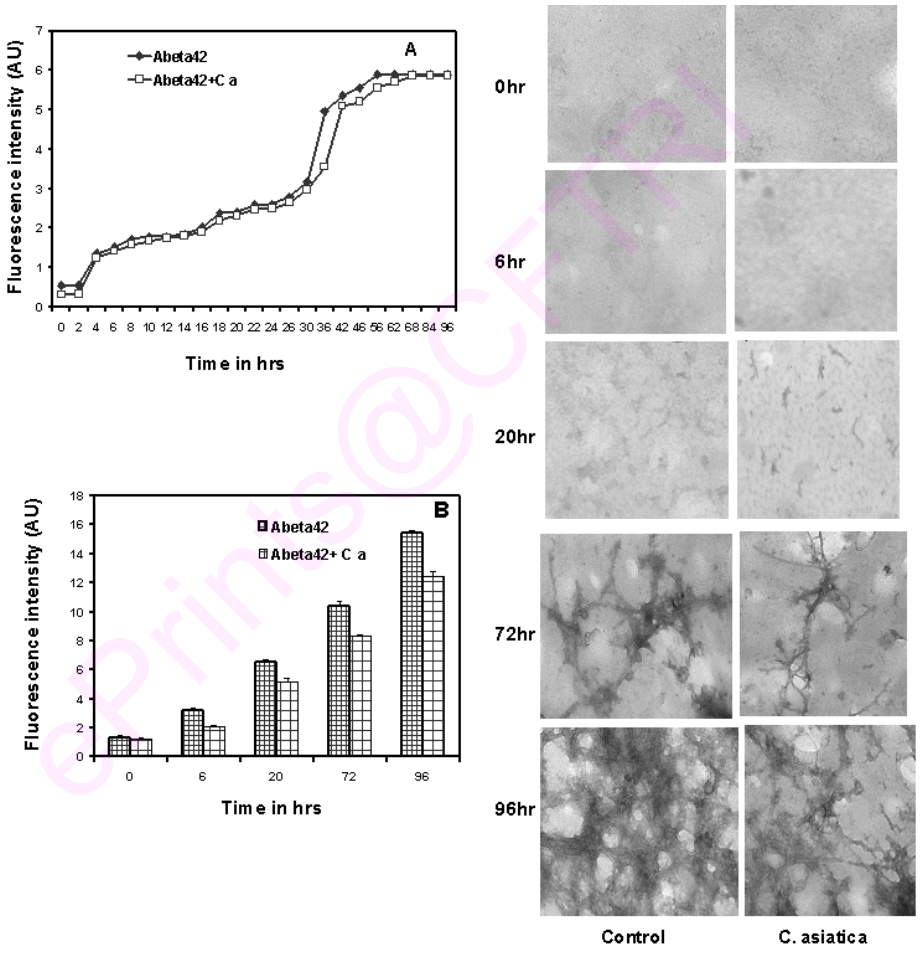


Fig 4C. 1. Effects of water leaf extract of C.asiatic on the prevention of amyloid fibrils from monomers (Phase I): A- The reaction mixture containing 50μM Aβ(1-42) co-incubated in absence or presence of 100 μg of extract at 37^{o}C in Tris-Cl buffer (pH 7.4). Aliquots are taken at 2 h intervals from 0-96 h. The fluorescence of thioflavin –T is recorded with excitation and emission wavelength of 446nm and 482nm respectively. B- Bar diagram showing the thioflavin-T fluorescence of Aβ(1-42) in presence or absence of *C. asiatica leaf* extract at 0, 6, 20, 72 and 96 h. C-

Transmission Electron Microscopic study: The reaction mixture contain 50μM Aβ(1-42) co-incubated in absence or presence of 100 μg of extract at 37^{o}C in Tris-Cl buffer (pH 7.4). The aliquots are taken at 0, 6, 20, 72 and 96 h and analyzed for presence or absence of aggregates.

4C.3. 2. Phase II: Inhibition of the formation of aggregates from oligomers: Fig 4C.2A shows the results of phase II. Thioflavin-T fluorescence data of Aβ is analyzed with and without extract at different time intervals (20, 36, 48, 72 and 96 h). Aβ is allowed to form oligomers for 20 h, and at 20 h, the extract is added and the Aβ aggregation process is monitored at different time intervals. The thioflavin-T fluorescence has increased from 20 to 96 h indicating the formation of fibrils from oligomers stage. In the presence of *C. asiatica*, the thioflavin-T fluorescence values do not decrease significantly indicating that the extract could not inhibit the formation of fibrils from oligomers. Fig 4C. 2B shows the results of TEM of phase II. The formation of fibrils increased with time from 20 to 96 h in Aβ alone and in the presence of *C. asiatica,* also the fibrils are seen but less in number. This indicates that *C. asiatica* could not be able to totally prevent fibril formation from oligomers (Fig 4C. 2B).

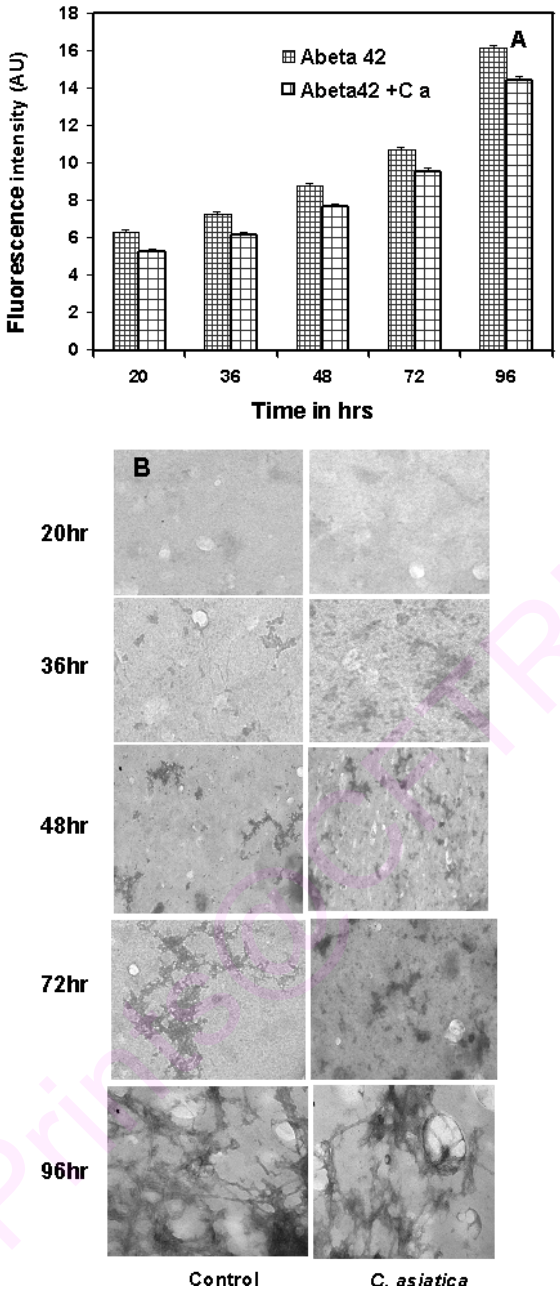


Fig 4C. 2: Effect of aqueous leaf extracts of C. asiatica on formation of amyloid fibrils from oligomers (phase II): A- Bar diagram showing the thioflavin-T fluorescence of Aβ(1-42) as a function of time: Aβ(1-42) is allowed to form oligomers for 20 h and *C. asiatica leaf* extract is added at 20 h of incubation. Aliquots are taken at 20, 36, 48, 72 and 96 h and thioflavin-T fluorescence emission was measured at 482nm. B-Transmission Electron Microscopy study: Aβ(1-42) is incubated for 20 h and *C. asiatica leaf* extract is added at 20 h of Aβ(1-42) incubation. Aliquots are taken at 20, 36, 48, 72 and 96 h. Aliquots are analyzed for the presence or absence aggregates using electron microscopy.

4C.3.3. Phase III: Dis-integration of pre-formed fibrils: Fig 4C.3A shows the results of thioflavin-T fluorescence assay of phase III. The matured fibrils after 96 h are allowed to further grow till 8 days and the fluorescence has increased from 16 AU to 28 AU respectively. However, in the presence of *C. asiatica* extract no significant reduction in thioflavin-T fluorescence was observed. This indicates that extract did not dis-integrate the pre-formed fibrils even after 8 days of incubation with *C. asiatica* extract. Fig 4C. 3B shows the results of TEM of phase III. At 96 h, there are matured fibrils with extensive branching in sample having Aβ alone. The extract is added to 96 h-matured fibrils and then incubated for 8 days. The extract could not dis-integrate totally the pre-formed fibrils even after 8 days of incubation.

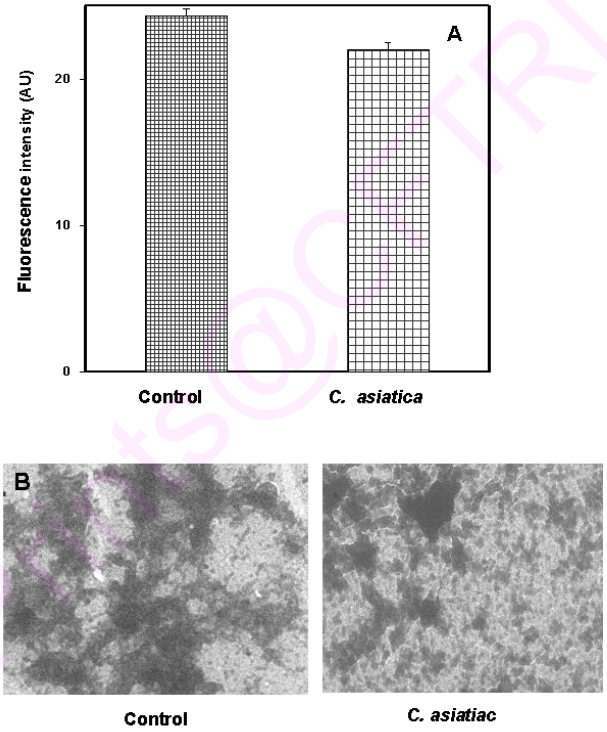


Fig 4C.3. Disintegration of preformed fibrils by water extract of *C. asiatica* (Phase III): A- Bar diagram showing the thioflavin-T fluorescence of Aβ(1-42): Aβ(1-42) is incubated for 96 h and *C. asiatica leaf* extract is added and further allowed to aggregate for 8 days. Thioflavin-T fluorescence measured at emission at 482nm. B-Transmission Electron Microscopy study: Aβ(1-42) is incubated for 96 h and then *C. asiatica leaf* extract is introduced and allowed to aggregate for 8 days. The aliquots are analyzed for the presence or absence of aggregates in electron microscopy.

4B.4. Discussion

Alzheimer's disease is a progressive neurodegenerative disease affecting millions of people worldwide. The etiological factors include oxidative stress, inflammation, Aβ over expression, elevation in metals etc. [Selkoe, 2001; Markesbery, 1997; Smith *et al*., 1997]. Among the risk factors implicated, Aβ is strongly associated with AD [Tanzi and Bertram, 2005 ; Tanzi and Bertram, 2001]. The therapeutic approaches in AD include, reducing the amyloid production or enhancing the clearance of amyloid load in AD [Koldamova *et al*., 2005; Walsh *et al*., 2005]. The ancient Indian system of medicine, Ayurveda has described traditional use of herbal medicinal therapies for the treatment of dementia [Manyam, 1999]. In particular, *C. asiatica* has been listed in ancient Indian Ayurveda medical text Caraka Susmita as a treatment for dementia. The leaves of *C. asiatica* is used as a memory booster in some regions of India [Kirtikar and Basu, 1993; Nadkarni, 1954]. The animal studies have shown that extracts of *C. asiatica* improves memory in rats [Kumar and Gupta, 2002; Gupta *et al*., 2003; Kumar *et al*., 2009]. The *C. asiatica* also found to improve memory, behavior and performance tests of mentally retarded children [Appa Rao *et al*. 1973]. Kumar and Gupta, [2003] have shown that *C. asiatica* prevents streptozotocin induced cognitive deficits in rats. Subathra *et al*., [2005] reported that *C. asiatica* reduced the protein carbonyls in the aged rat brain. Further, Kumar *et al*. [2009] have demonstrated that *C. asiatica* significantly decreases the acetylcholine esterase activity in colchicine induced cognitive impairment and oxidative stress. Nalini *et al*. [1992] reported that *C. asiatica* showed improvement in the avoidance task in rats. Rao *et al*. [2005] reported that *C. asiatica* able to improve the brain function of mice if treated during postnatal period. Recently, Dhanasekaran *et al*.[2009] have reported that *C. asiatica* moderately decreased Aβ(40) and Aβ(1-42) load both in cortex and hippocampus region of PSAP Alzheimer's disease mice model. But the reduction of Aβ(40) and Aβ(1-42) did not improve the Y-maze and open field behavior tests. The mechanism of reduction in the amyloid load is not clearly understood. There are no studies to show the effects of *C. asiatica* on Aβ aggregation kinetics. Our results showed that C. *asiatica* aqueous extract could not significantly inhibit the Aβ aggregation either from monomer or oligomers, and also the extract was not able to

dis-integrate the preformed fibrils. Our results and the observations made by others can be explained by the following hypothesis. Aβ exists in monomer form and monomers will be in random coil conformation. Aβ in suitable condition self-aggregates into fibrils. The aggregation process will pass through different conformation in the following order: random coil, misfold, β-sheet/ β- turn or in combination of all these conformations. The fully aggregated long fibrils will be either in β- sheet/ β- turn conformation. The aqueous extract of *C. asiatica* could not stabilize random coil of monomers hence could not prevent fibril formation. However, we further propose that *C. asiatica* extract may be acting by other possible pathways such as (i) enhancing the α- secretase pathway of APP processing or inhibiting β-secretase activity; (ii) may be acting as anti-oxidant, so that oxidative stress will be reduced. And Oxidative stress enhances Aβ expression; (iii) may enhance clearance mechanism of accumulated amyloid; or (iv) may be acting as anti-inflammatory candidate. All these events together may be helpful for *C. asiatica* as brain tonic or memory or cognitive function enhancer. Further work is needed to understand more on the efficacy of *C. asiatica* as a therapeutic intervention molecule.

Chapter 5

General Summary and Conclusions

Chapter 5 General Summary and Conclusion

5.1. General Summary and Conclusion

Alzheimer's disease (AD) is the most common form of dementia and affects one in four individuals over the age of 85. AD has multiple etiological factors, which includes genetics, environmental factors, and lifestyle. The hallmark pathology of AD includes extracellular amyloid β protein (Aβ) deposition in the form of senile plaques and intracellular deposits of the microtubule-associated protein tau as neurofibrillary tangles in the AD brain. The diagnosis of AD is based on the characteristic idiopathic psychometric deficits upon clinical evaluation and further confirmed by post-mortem due to the presence of the characteristic lesions described above. Still AD pathology for neuronal cell death and factors responsible for AD onset are not clear. Further, there are no reliable biomarkers for the early detection of AD. MRI is the only hope to confirm AD atrophy for diagnosis purpose, but not for early detection yet. This made the drug discovery for AD a touch challenge. The over expression of Amyloid β peptide and accumulation of the same as intracellular and extracellular aggregates is agreed major pathological event in AD and the decrease in amyloid load is a drug target for AD. Aβ is produced by sequential proteolytic processing of a larger Aβ protein precursor (AβPP) by β-secretase to generate a large secreted fragment sAPPβ and a 99 aa cellular fragment – CTFβ – that includes Aβ, the transmembrane domain and the intracellular domain of AβPP. But there multiple pathways which influence Aβ production.

The accumulation of Aβ in the form of senile plaques is found to correlate with neuronal loss and brain atrophy and ventricular expansion. The brain atrophy is correlated with cognitive decline in AD. The studies with AD patients showed a positive correlation between amyloid load and brain atrophy. The significant contribution towards *in vivo* quantification of amyloid came through the development of Benzathiol derivative, N-methyl [11C] 2-(4'-methyl aminophenyl)-6-hydroxy-benzothiazole (PIB), which specifically bind to amyloid and can be imaged in Positron Emission Tomography (PET) scan. The specificity of PIB is that it only binds to aggregated forms of Aβ and not to monomeric forms of Aβ. The toxicity of

Aβ is depends on form of Aβ namely oligomer or fibril form. The recent understanding on amyloid hypothesis suggests that oligomeric form of Aβ is more toxic than fibrils. The literature on genotoxicity of amyloids is scanty. The available reports on genotoxicity of amyloids suggest that amyloids causes DNA damage mainly through oxidative stress and free radicals. However there are reports on the translocation of Aβ in to nuclear region. The earlier report suggested the presence of aggregated Aβ(1-42) in the nucleus of CHO cells and also in the nuclear region of AD brain sample. The role of Aβ in nucleus is not known. The earlier studies from our lab have shown that Aβ(1-42) binds to DNA and changes DNA stability and helicity. Also our lab earlier provided evidence that Aβ has DNA nicking ability. The role of Aβ(1-42) in genotoxicity is not clear. Hence the present study is aimed to understand the DNA nicking ability of different fragments of Aβ and *in vivo* studies to understand the Aβ(1-42) induced genotoxicity. And also, the role novel indigenous plants such as *C. crista* and *C. asiatica* extract in preventing Aβ induced DNA damage and preventing Aβ aggregation. The present study has following objectives.

3. To study mapping of amyloids induced genotoxicity.
4. In vivo studies using aged rabbits to understand the neuroprotective role indigenous plants in amyloids induced genotoxicity in relevance to brain tomography and DNA damage.

3. To study anti-amyloidogenic properties of indigenous medicinal herb

Chapter 1: General Introduction

The general introduction covers the etiology and pathology of AD. Also the biochemistry behind the neuronal cell death. The introduction focussed on the role of amyloid in AD neurodegeneration and also the role dietary factors in AD. The following of nut shell covered in introduction.

1. Alzheimer's disease (AD) is the most common form of dementia and affects one in four individuals over the age of 85.

2. The hallmark pathology of AD includes extracellular amyloid β protein (Aβ) deposition in the form of senile plaques and intracellular deposits of the microtubule-associated protein tau as neurofibrillary tangles in the AD brain.

3. DNA instability has been implicated in the pathogenesis of neurodegenerative disorders including Alzheimer's disease, amyotrophic lateral sclerosis, Down's syndrome, Parkinson's disease, etc

4. The studies with AD patients showed a positive correlation between amyloid load and brain atrophy.

5. AD has multiple etiological factors including genetics, environmental factors, and general lifestyles.

6. Several cross-sectional studies suggest a relationship between particular nutrients in diet and the presence of cognitive changes in AD.

7. A number of dietary factors such as saturated fatty acids, higher calorie intake, and excessive alcohol have been have indicated that diet plays a role in AD.

8. In contrast, antioxidants, fish, methionine-rich proteins, and vitamins are identified as protective against the disease.

9. Calorie restriction is reported to slow the progression of neurodegeneration in AD.

10. The diet rich in docosahexaenoic acidis may reduce the risk of developing AD.

11. Though vitamin E is reported to reduce the risk of AD, the dietary supplements of vitamin E fail to provide better results compared to dietary intake of vitamin E.

12. Hyperhomocysteine levels induce neurologic abnormalities such as cerebral atrophy, and seizures.

13. A deficiency of vitamins is found to elevate the concentration of homocysteine, which is implicated in vascular mechanisms leading to AD.

14.Folate and vitamin B12 are involved in biosynthesis of methionine from its precursor homocysteine, whereas, B6 has a role to play in the conversion of homocysteine to cysteine.

15.The green tea flavanoids, EGCG has recently been shown to have neuroprotective functions such as antioxidation, iron chelation, and anti-inflammation.

16.Several epidemiological studies have shown that moderate wine consumption reduces the risk of developing AD and these effects are attributed to the resveratrol in the wine.

17.The curcumin present in the turmeric is reported to reduce the risk of developing AD by its anti-oxidant, anti-inflammatory and anti-amyloidogenic activities.

18. Diet-genetic interactions may play an important role in healthy aging and in AD.

19.The drug discovery programs in AD targeted the beta-amyloid mainly and others include metals, inflammatory molecules, oxidative stress and etc.

The introduction ended with objectives.

Chapter 2: Studies to map amyloidogenic peptides induced genotoxicity

Alzheimer's disease (AD) is a common form of dementia and it is characterized by loss of memory, inability to perform daily activities, language impairment and behavioral abnormalities. Aβ implicated in AD causes neurotoxicity through neuronal death and it is demonstrated *in vitro* through apoptosis of neuronal cell. This current study is undertaken to understand the mechanism of Aβ fragments induced genotoxicity.

1. The results showed that Aβ fragments (1-11, 1-28, 1-40 and 1-42) nick ScDNA and causes the open circular and linear forms. Aβ fragments (22-35, 25-35, 17-42 and 1-43) do not nick ScDNA.

2. The Mg^{2+} enhanced DNA nicking ability of Aβ fragments (1-11, 1-28, 1-40 and 1-42) and other Aβ fragments (22-35, 25-35, 17-42 and 1-43) also showed DNA nicking activity in presence of Mg^{2+}.

3. The histidine modified Aβ(1-11) do not nick ScDNA. Also, histidine modified Aβ (1-11) retarded λ-DNA, single stranded circular and double stranded circular DNA mobility compared to unmodified Aβ(1-11) but could not cause nicking.

4. The nuclease inhibitor, Aurin tricartboxylic acid inhibited only Aβ(1-42) induced DNA nicking and could not inhibit Aβ(1-11,1-28,1-40) induced DNA nicking in the presence of Mg^{2+}.

6. The melting temperature and ethidium bromide binding studies showed that DNA became unstable in presence of different fragments of Aβ as evidenced by decrease in Tm and EtBr binding.

7. The Circular Dichroism studies on the effect of histidine modified Aβ(1-11) on ScDNA conformation showed slight change in the ScDNA conformation.

8. The Circular Dichroism study on Aβ (1-11, 1-28, 22-35, 25-35 and 1-43) fragments secondary conformation showed they are in random coil conformation.

9. The Protscale study insighted the contribution of different amino acid residues to secondary conformation.

Chapter 3: In vivo studies using aged rabbits to understand the neuroprotective role Indigenous plants in amyloids induced genotoxicity in relevance to brain tomography and DNA damage.

The present study focused on Aβ(1-42) induced genotoxicity in aged rabbits and its relation to MRI changes in brain and also the effects plant extracts and curcumin derivatives in modulating Aβ(1-42) induced DNA damage (3A)
The following are the significant findings.

Chapter 3A-a: Aβ(1-42) induced genomic instability in aged rabbit brain

1. The present study is aimed to understand the *in vivo* effects of Aβ(1-42) on genomic DNA stability and conformation and its relevance to brain atrophy.

2. The aged (4 yr) New Zealand rabbits are intracisternally injected with Aβ(1-42) and sacrificed after 25 days, when the rabbits developed AD like behaviour.

3. Genomic DNA is isolated from frontal cortex (FC), hippocampus (H) and midbrain (M) regions of Aβ(1-42) injected and control aged rabbit brain.

4. DNA stability parameters are analyzed and the results showed that DNA is damaged in FC and H; where as in M, DNA is in condensed state.

5. The DNA conformation study evidenced the presence of C, π and ψ- type DNA conformations in FC, H and M of Aβ(1-42) injected rabbit brain regions respectively. But in control rabbit brain, DNA is in B- conformation in all the brain regions studied.

6. Magnetic resonance imaging (MRI) studies showed that there are no significant changes in brain structures between control and Aβ(1-42) injected aged rabbit brain regions at 25 days.

Chapter 3A-b Inhibition of Aβ(1-42) induced DNA damage by Indigenous plant extract

1.The protective role of curcumin and its derivatives and aqueous leaf extracts of *Caesalpinia crista* and *Centella asiatica* against Aβ(1-42) induced DNA nicking is studied. The results showed that curcumin derivatives and *Caesalpinia crista* and *Centella asiatica* could not prevent Aβ(1-42) DNA nicking activity.

2.The Circular Dichroism studies on the effect of curumin glucoside (CG) and vanillin glucoside (VG) on Aβ(1-11), Aβ(25-35) and Aβ(1-28) conformation showed a differential effects. The CG increases the beta sheet conformation of Aβ(1-11), with a corresponding decrease in the random coil and not significant change in the alpha helix quantity. But VG increases alpha helix and beta sheet and correspondingly decreased in the random coil conformation.

3. The CG and VG increase the beta sheet conformation of Aβ(1-28) and Aβ(25-35) corresponding decrease in random coil conformation while no change in the alpha helix conformation.

Chapter 3B: Aβ(1-42) induced brain MRI changes in aged rabbits resembles Alzheimer's disease

1. The present study is undertaken to understand whether Aβ(1-42) induced brain atrophy in aged rabbit brain resembles MRI changes in AD brain.

2. Ten aged (4 yrs) rabbits are injected with 100μg of Aβ((1-42) through intracisternal route and ten control aged rabbits are injected with saline. Rabbits are subjected to 1.5T MRI scanning before and after 45 days of Aβ injection. The time course of 45 days is chosen as rabbits developed AD like symptoms such as forward head tilting, hemiplegic gait, loss of appetite, isolation behaviour, splaying of extremities and paralysis. We also conducted MRI studies on 5 normal and AD patients.

3. We found that there are significant reduction in the thickness of hippocampus, temporal lope, frontal lobe, and midbrain and an increase in lateral ventricle volume.

4. All these significant changes indicate that Aβ(1-42) induced brain atrophy mimics AD brain atrophy.

Chapter 4: To study anti-amyloidogenic properties of indigenous medicinal herb

The present focussed in evaluating the anti-amyloidogenic potential of indigenous medicinal herb using a novel in vitro battery of tests. The results indicate the following significant results.

Chapter 4A: Anti-oxidant and anti-inflammatory properties of *Caesalpinia crista* and *Centella asiatica*

1. In the present study, antioxidant properties of aqueous leaf extract of *Caesalpinia crista* and *Centella asiatica* is carried out using, DPPH assay, reducing potential assay and superoxide assay.

2. The anti-inflammatory activity of *Caesalpinia crista* and *Centella asiatica* are demonstrated using 5-lipoxygense assays as 5-lipoxygense involved in inflammation.

3. The *Caesalpinia crista* and *Centella asiatica* dose dependently scavenged $DPPH^{\bullet}$ and the IC_{50} of *C. crista* less than *C. asiatica*. Both the extracts showed more IC_{50} compared to standard ascorbic acid.

4. The superoxide assay showed that *C. asiatica* and *C. crista* dose dependently scavenged the superoxide anion radical. The IC_{50} of *C. asiatica* is lesser than *C. crista*. But both the extracts showed IC_{50} more than the standard, ascorbic acid.

5. The reducing potential assay indicates that *C. crista* has more reducing potential compared to *C. asiatica*. Both the extracts showed lesser reducing potential compared to standard, ascorbic acid.

6. The 5-lipoxygense inhibition assay showed both the extracts dose dependently inhibited the enzyme and *C. crista* effectively inhibited compared to *C. asiatica.*

Chapter 4B: Antiamyloidogenic property of leaf aqueous extract of *Caesalpinia crista*

1. In this study, we aim to understand the effect of leaf aqueous extract of *C crista* in the prevention of Aβ(1-42) aggregation.

2. We have used three key battery tests to understand the effect of *C. crista* in preventing the formation of, (i) aggregates from monomers (ii) aggregates from oligomers and also (iii) dis-integration of preformed fibrils of Aβ.

3. The *C. crista* aqueous extract could effectively found to inhibit the Aβ(1-42) aggregation both from monomer and oligomers. Also, the extract is able to dis-integrate the preformed fibrils.

5.The anti-amyloidogenic property of *C. crista* may be attributed to the water soluble polyphenols present in the aqueous extract.

Chapter 4C: Studies to understand the effect of *Centella asiatica* on Aβ(1-42) aggregation in *vitro*

1. The present study, we have used Aβ(1-42) for the formation of oligomers, protofibrils and fibrils and to map *C. asiatica* for its anti-Aβ(1-42) aggregation property.

2. We focused on, (i) whether the *C. asiatica* leaf aqueous extract prevent the formation of oligomers and aggregates from monomer?; (ii) Whether the C. *asiatica* aqueous extract prevent the formation of fibrils from oligomers; (iii) whether the aqueous extract dis-aggregates pre-formed fibrils.

4.The C. *asiatica* aqueous extract could not significantly inhibit the Aβ(1-42) aggregation either from monomer and oligomers and also not be able to dis-integrate the preformed fibrils.

5.2. Significant Conclusion

The data on Aβ fragments (1-11, 1-28, 1-40 and 1-42) induced DNA nicking indicates, different mechanism of ScDNA nicking by different Aβ fragments. The involvement of histidine of Aβ(1-11) in DNA nicking insight a new role of Aβ as nuclease. The differential effects of different Aβ on DNA stability parameters indicates complex behaviors of these Aβ fragments.

The *in vivo* effects of Aβ(1-42) in aged rabbits suggests that causes changes the DNA stability and helicity. The Aβ(1-42) alters DNA conformation in aged rabbit brain region specifically. It changes B-DNA conformation of FC, H and M to C-type, π and ψ-DNA conformation respectively. But changes in the DNA stability and DNA conformation do not induce brain atrophy in 25 days of Aβ(1-42) injection. The Aβ(1-42) injection to aged rabbits and allowed for 45 days has showed brain reduction thickness of frontal lobe, hippocampus, midbrain, temporal lobe and increase in lateral ventricle volume compared to control rabbit brain regions. These brain regions thickness and volume changes are comparable with AD patients brain thickness changes.

The aqueous leaf extracts of *C.crista* and *C.asiatica* showed both anti-oxidant and anti-inflammatory activities. These activities are attributed to the water soluble polyphenols in the extract. So these extracts are further used to understand the anti-amyloidogenic property. The *C. crista* not only inhibited the formation of amyloid fibrils both from monomers and oligomers but also dis-aggregated the pre-formed

fibrils. The anti-amyloidogenic property of C.crista is attributed to water soluble polyphenol present in the extract. While *C.asiatica* neither prevented the formation of amyloid fibrils both from monomers and oligomers nor dis-aggregated the pre-formed fibrils. This data on *C.crista* is promising to be drug candidate for AD.

These investigations provided newer understanding on the role of Aβ(42 in AD neurodegeneration as indicated by advanced studies like MRI, genomic stability and protein aggregation.

Chapter 6

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Publications of the Candidate

List of candidate publications

I. Research Articles Published:

(1) Ramesh BN, Keshav Raichurkar P, Shama Sundar NM, Basavaraj KH, Rao TSS, Sambamurti K, Rao KSJ, Molecular studies on A□(42) induced genomic instability in aged rabbit brain. Neurochemistry International (2010) 56, 655-662.

(2) Ramesh BN, Indi SS, Rao KSJ. Studies to understand the effect of *Centella asiatica* on A□(42) aggregation in *vitro.* Current Trends in Biotechnology and Pharmacy (2010) 4(2), 716-724.

(3) Ramesh BN, Indi SS, Rao KSJ. Anti-amyloidogenic property of leaf aqueous extract of *Caesalpinia crista*. Neuroscience Letters (2010) 475(2), 110-114

II. Review Articles Published:

(1) Rao TSS, **Ramesh BN**, Vasudevaraju P, Rao KSJ Molecular biology research in neuropsychiatry: India's contribution. Indian J Psychiatry, (Supplement) (2010) 52(1), S299-S306**.**

(2) Ramesh BN, Rao TSS, Prakasm A, Sambamurti K, Rao KSJ, Neuronutrition and Alzheimer's disease. Journal of Alzheimers Disease (2010) 4, 1123-1139

(3) Rao TSS, Asha MR, **Ramesh BN**, Rao KSJ, Understanding nutrition, depression and mental illness. Indian Journal of Psychiatry (2008) 50, 77-82.