Screening and evaluation of natural compounds for therapeutic application in Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is one of the most common causes of dementia, which is affecting more than 26 million people, globally. It is an irreversible, progressive brain disease that slowly destroys cognitive memory. The major pathophysiology of Alzheimer's disease is deposition of senile plaques (Amyloid Plaques) and neurofibrillary tangles (NFTs) in the brain causing neuronal damage, ultimately leading to the loss of cognitive function and memory loss. Amyloid peptide (Aβ), a major component of amyloid plaques, is the product of proteolytic cleavage of the amyloid precursor protein (APP). APP is acted upon by 2 different enzymes namely, β-secretase (BACE1) and γ-secretase in a sequential order, which results in the production of $A\beta_{1-40}$ and $A\beta_{1-42}$ fragments. It is identified that the imbalance between the formation and clearance of Aβ peptides leads to their subsequent aggregation and plaque formation in the brain leading to Alzheimer's disease. BACE1 is one of the major therapeutic targets for the treatment of Alzheimer's disease. Currently, there are no drugs available in the market neither for complete cure nor for the symptomatic relief of Alzheimer's disease. Currently available drugs can prolong the onset of the disease or delay the disease progression. Although they are efficient in reducing the disease burden to some extent, side effects associated with these drugs limit their long term use. Several synthetic drugs failed during their development as treatment due to lack of efficacy and/or toxicity. The current information on Alzheimer's disease treatment clearly points out that, there is a need for therapeutic intervention which can reduce the burden of the disease with minimal or no side effects.

The possible alternative solution to present day synthetic drugs with limited efficacy and serious side effects would be to select the right drug target and considering the compounds from natural sources. Selection of right drug target is very important in the development of therapeutic interventions for Alzheimer's disease, which can lead to efficient drugs with disease cure potential. Natural molecules from plants and animals are used for human therapeutic applications from time immemorial and are found to be safe for human consumptions and also effective in treating several human diseases. Diversity in chemical structure and bioactivity makes phytochemicals as most preferred

choice for screening of therapeutic molecules. Many of such natural compounds/phytochemicals are known to have neuroprotective and memory enhancing activity but the mechanism is unknown. In the current study, natural molecules were screened against a validated drug target and evaluated for potential therapeutic efficacy for the treatment of Alzheimer's disease.

The research objective of the current doctoral thesis work was mainly aimed at finding a potential drug candidate from natural sources to treat Alzheimer's disease with no or minimal toxicity. The key objective was to screen natural molecules against one of the validated Alzheimer's disease drug target called BACE1 using *in silico* tools and further validate the efficacy of screened molecules through *in vitro* and *in vivo* studies.

Phytochemicals for *in silico* screening were selected based on the traditional knowledge available on the herbs. Phytochemicals, which are traditionally known for their effects on memory and related applications, were short-listed for preliminary screening. Based on traditional knowledge, 47 neuroprotective natural compounds (phytochemicals) were selected and docked against BACE1 as a target protein. Two natural compounds were short-listed for further evaluation based on *in silico* screening results. Curcumin and one of the metabolites of Curcumin, called Bis-demethyl curcumin (BMC) were selected and considered further to evaluate the efficacy in *in vitro* and *in vivo* studies.

In *in silico* screening, AutoDock was used for screening potential phytochemicals based on its binding energy (affinity) with BACE1. Molsoft and Osiris software's were used for further characterizing and predicting drug like properties of selected compounds. Screened phytochemicals showed varied binding energy ranging from -5.16 to -21.41 kcal/mol. Curcumin and BMC were selected for evaluation of molecular properties and drug-like properties, in which BMC was found to have higher drug score.

In *in vitro* evaluation, antioxidant, anti-inflammatory and BACE1 inhibitory activity of the short-listed compounds were carried out. BACE1 inhibition activity of BMC was undertaken using FRET assay. BMC was shown to have several fold higher antioxidant activity when compared to Curcumin and reference compound Vitamin C in all the antioxidant assays. Also, it was found to be superior in anti-inflammatory activity when compared to Curcumin. BMC inhibited pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β with the IC50 values of 88.27, 119.9 µg/ml and 26.94 µg/ml respectively

and were lower than that of Curcumin. BMC inhibited 5-Lipoxygenase enzyme with the IC_{50} value of 6.8 µg/ml and was 4.5 times lesser than that of Curcumin (27.47 µg/ml). Additionally, BMC exhibited very good BACE1 inhibitory activity with IC_{50} value of 1.471 µg/ml. On the whole, BMC has shown multipotent activity comprising antioxidant activity, anti-inflammatory activity and BACE1 inhibitory activity.

Since, BMC was found to be superior to Curcumin in *in vitro* studies, it was further evaluated in aluminum chloride-induced animal model of Alzheimer's disease. BMC was found to have poor aqueous solubility, which may be linked to its poor bioavailability. Hence a suitable formulation was developed to improve its aqueous solubility, which would increases the bioavailability of BMC *in vivo*. The developed BMC formulation was further evaluated for the efficacy in aluminum chloride-induced animal model study.

Alzheimer's disease was induced in Sprauge-Dawley rats by intraperitonial administration of aluminium chloride. In this animal model, various parameters were investigated which includes, estimation of lipid peroxidation (LPO) in the brain, superoxide dismutase (SOD) activity in the brain, circulating superoxide dismutase activity in blood, protein phosphatase 2A (PP2A) activity. In addition, histopathology of the brain was performed in order to locate the probable sites of degeneration. Lipid peroxidation was found to be high in the disease group implicating increased oxidative stress upon administration of aluminum chloride. In BMC group, Lipid Peroxidation (LPO) was significantly reduced as compared to the disease control ($P<0.05$). Also, BMC significantly increased the levels of SOD in brain, plasma and in RBC as compared to disease group ($P<0.05$). No significant difference was observed between LPO and SOD values of the control *versus* BMC group. The SOD level in BMC group was found to be comparable to the control group indicating antioxidant property of the BMC in the developed animal model. Also, in BMC group PP2A activity was significantly increased as compared to disease group. PP2A activity of the BMC group was comparable to control group. Increased PP2A activity in BMC group might be attributed to the reduced hyperphosphorylation of tau and consequently reduced neurofibrillary tangles (Walton, 2007), thus decreasing neurological damage induced by aluminum chloride, upon treatment with BMC. Nevertheless, BMC was found to be

effective neuroprotective compound in aluminium chloride-induced Alzheimer's disease model of rat.

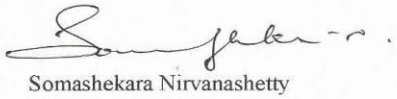
In conclusion, the research work carried out suggested that BMC is a multipotent and efficacious neuroprotective molecule, which can be further considered for the development as a drug for the treatment/management of Alzheimer's disease. Moreover, BMC is a safe and effective multitarget drug candidate for the treatment of Alzheimer's disease, which can be further developed into a drug as per the regulatory requirement and commercialized.

Declaration

Declaration

This thesis is the result of my own work and to the best of my knowledge, includes nothing, which is the outcome of work done in collaboration except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university of institution for any degree, diploma, or other qualification.

Signature:



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Nomenclature

AD	Alzheimer's disease
Aβ	Beta Amyloid
ABTS	2, 2-azinobis (3-ethylbenzothiazoline-6-suifonic acid)
ACH	Acetylcholine
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
AD-TNDCI	Alzheimer's Disease -Type Neurodegeneration with Cognitive Impairment
ALCAR	Acetyl-L-Carnitine
AMPA	Alpha-Amino-3-Hydroxy-5-Methyl-4-isoxazole Propionic acid
APO E4	Polio Protein E4
APP	Amyloid Precursor Protein
Asp2	Membrane-bound aspartyl protease
BACE1	β-site APP cleaving enzyme 1 (Beta-Secretase 1)
BBB	Blood Brain Barrier
1BCP	1-(1, 3-Benzodioxol-5-yl Carbonyl)-Piperidine
BMC	Bis-Demethyl Curcumin
Ca	Calcium
CGA	Chlorogenic Acid
ChEIs	Cholinesterase Inhibitors
CHOLEST.	Cholesterol
CMC	Critical Micellar Concentration
CNS	Central Nervous System
COX	Cyclooxygenase

CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CSF	Cerebrospinal Fluid
CWS	Cold Water Stress
CYP	Cytochrome P450
3D	3 Dimensional
Da	Dalton
DNA	Deoxy Ribonucleic Acid
DPPH	2,2-Diphenyl-1-Picryl-Hydrazyl-Hydrate
EPIDIDY.	Epididymis
ER	Endoplasmic Reticulum
FDA	Federal Drug Administration
FGF	Fibroblast Growth Factor
FMO	Flavin-containing Monooxygenase
FRAP	Ferric Reducing Anti-oxidant power
FRET	Fluorescence Resonance Energy Transfer
GABA	Gamma-Amino Butyric Acid
GABA-T	GABA Transaminase
GABAa	Gamma-Amino Butyric Acid (GABA) type A receptor
GABAb	Gamma-Amino Butyric Acid (GABA) type B receptor
GABAc	Gamma-aminobutyric acid (GABA) type C receptor
GOLD	Genetic Optimisation for Ligand Docking
GSK	Glycogen Synthase Kinase-3
GST	Glutathione-S-Transferase
G-sec	Gamma-Secretase
Hb	Haemoglobin
6-HF	6-Hydroxy Flavone

4-HNE	4-Hydroxynonenal
H2O2	Hydrogen Peroxide
HTS	High Throughput Screening
5-HT	5-Hydroxytryptamine
IC50	Inhibitory Concentration
IgG	Immunoglobulin-G
IL-1	Interleukin 1
iNOS	Inducible Nitric Oxide Synthase
ISF	Brain Interstitial Fluid
JNK	Jun-N-Terminal Kinases
LOX	Lipoxygenase
LPO	Lipid Peroxidation
LRP1	Low-Density Lipoprotein Receptor-related Protein
Lympho.	Lymphocytes
MAPK	Mitogen-Activated Protein Kinases
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
Mg	Magnesium
mRNA	Messenger RNA
MTC	Methylthioninium Chloride
NAP	Neuronal Tubulin-Preferring Agent
Neutro.	Neutrophils
NFTs	Neurofibrillary Tangles
NGF	Nerve Growth Factor
NINCDS Stroke	National Institute of Neurological and Communicative Disorders and

NMDA	N-Methyl-D-Aspartate
NOS	Nitric Oxide Synthetase
NRG	Growth Factor Neuregulin
NSAIDs	Nonsteroidal Anti-inflammatory
PBPK	Physiologically Pharmacokinetics
PC 12	Pheochromocytoma Cells
PCV	Packed Cell Volume
PD	Parkinson's Disease
PHF	Paired helical Filaments
PKC	Protein Kinase C
PP2A	Protein Phosphatase 2A
PPAR-γ	Peroxisome Proliferator-Activated Receptor Gamma
PS1	Presenilin 1
PS2	Presenilin 2
p-tau	Phosphorylated Tau
RBC	Red Blood Cell
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RYRs	Ryanodine Receptors
sAPPβ	Amino terminal ectodomain fragment of APP
Ser	Serine
S.CREAT	Serum Creatinine
SGPT	Serum Glutamyl Pyruvate Transaminase
SGOT	Serum Glutamyl Oxaloacetate Transaminase
SH-SY5Y	Neoroblastoma Cells
SOD	Superoxide Dismutase

SULT	Sulfotransferase
S.UREA	Serum Urea
SW	Swedish
Tg	Transgenic
TGF-b1	Transforming Growth Factor, Beta 1
TG3	Tau Antibodies
Thr	Threonine
TRYGLY.	Triglycerides
TX67	Taxane Analog
UGT	Uridine Diphospho-Glucuronosyl Transferase
50-UTR	50-Untranslated Region
WBC	White Blood Cells
WHO	World Health Organization

Chapter-1

1. Introduction to the Research Work

1.1. Alzheimer's disease – Description:

Alzheimer's disease is one of the most common causes of dementia in the world (Castro *et al.,* 2010; Bajda *et al*., 2011; Anand *et al.,* 2014). It is a multifactorial, chronic and progressive neurodegenerative disorder characterized by cognitive, behavioral and functional impairment (Castro *et al.,* 2010). Alzheimer's disease is mainly associated with age and the incidence rate of Alzheimer's disease increases exponentially with age (Anantharaman *et al.,* 2006; Banks, 2012). The disease is characterized by loss of memory and cognitive functions impairing day to day life (Humpel, 2011). There are several symptoms, seen as a warning for a developing Alzheimer's disease such as mood changes, memory loss, difficulty in carrying out simple tasks, misplacing things, poor judgment, problems with language, and finally personality changes (Hardy, 2002). It is a growing health crisis around the world, having affected 33.9 million people worldwide (Barnes and Yaffe, 2011) and it is estimated that by 2050, nearly 80 million people would be affected by this disease worldwide (Humpel, 2011). Though Alzheimer's disease has been extensively studied for decades, there is no cure for Alzheimer's disease (Swaminathan *et al,* 2014).

The term Alzheimer is given after the German psychiatrist Dr. Alois Alzheimer, who reported the first case of what was named later as 'Alzheimer's disease' in 1906. Very little is known about the predisposing factors, pathway of neuronal damage and battery of immune responses reacting to the neuronal abnormalities. The earliest symptom is memory loss and short term recall, particularly in the areas in the hippocampus region of the brain, where memory is stored. Preclinical progress starts in the entorhinal cortex, which is connected to the hippocampus, which is responsible for memory formation (Geldmacher *et al*., 1997). Later, communicate between the neurons will be lost and they die subsequently (Singh, 1997). There are three stages of developmental transition in Alzheimer's disease, *viz*., early stage, middle stage and late stage. Early stage is

found in individuals of any age and the symptoms are mild cognitive impairment such as changes in mood and behavior, forgetfulness and verbal and written communication problems. There will be sharp decrease in cognitive functions in the middle stage of the disease (Small *et al*., 1997). In the late stage, individual cannot communicate verbally or look after themselves (Bergman *et al*., 1997), totally depending on family members or caretakers.

1.1.1. Categories, Pathophysiology and Etiology of Alzheimers disease:

There are two main categories in Alzheimer's disease and are called as Familial and Sporadic. Familial type is a disease, which is transmitted from one generation to next through vertical gene transfer. Only five percent of the total Alzheimer's disease is caused by this type and the rest is by Sporadic that occurs randomly in the population. The pathology of Alzheimer's disease is categorized on a macro level in a progressive way for memory loss (Schofield *et al.,* 1997). Alzheimer's disease is divided further into early onset, where the onset of the disease occurs at the age of below 65 years and secondly late onset, where the disease is reported to occur after the age of 65 years. Hence there are four different subtypes of Alzheimer's, which are as follows; early onset familial, late onset familial, early onset sporadic and late onset sporadic (Marx, 1996; Forsyth, 1998). More rapid deterioration occurs in cognitive and physical abilities during the early onset stage when compared to the late onset stage, however, some of the individuals do not exhibit all kinds of symptoms and signs in each stage (Larson *et al*., 2006). During the early stage, an individual exhibits mild symptoms, which are difficult to identify and often overlooked. As the later stage progresses, the severity of the symptoms increases to an extent, which causes the individual to be totally dependent on others. Some of the symptoms like loss of long term memory, motor skills and the ability to learn new procedures can be seen until the last stage (Forsyth, 1998; Podewils *et al.,* 2005). The neurons die in a particular pattern over time as the disease progresses (Schofield *et al*., 1997) leading to cognitive impairment (Orr *et al.,* 2002).

In Alzheimer's disease patients, neuronal loss generally starts even before the sign of memory loss can manifest. Cerebrospinal fluid gradually fills the space, which was occupied earlier by brain tissue due to the progression of brain atrophy (Small *et al*., 1997). The patient starts experiencing a decline in the ability to process complex

thoughts, then progressing towards mood and personality changes. The atrophy of the brain spreads to the other areas of the cerebral cortex. As the disease progresses, the atrophy affects the areas, which control speech, sensory perceptions, reasoning and thinking ability. A number of other cerebro-vascular abnormalities like decreased microvascular density, basement membrane thickening and decreased glucose transport across the blood brain barrier, to name a few, have also been observed (Farkas and Luiten, 2001). Women have greater chances of getting Alzheimer's disease due to the increased risk factors during postmenopausal period. Early onset of Alzheimer's disease occurs in the individuals with a history of head damage associated with conscious loss (Dewji *et al*., 1996).

During the past decade, tremendous progress has been made in determining the multifactorial etiology of Alzheimer's disease (Jun *et al.,* 2012). There are several risk factors, which are involved in the development of the Alzheimer's disease (Schofield, 1997). Such risk factors of Alzheimer's disease include age and genetic disorders, whereas the other factors are history of the head injury, exposure to toxins, metals and finally, family history (Oakley, 1993). The percentage of Alzheimer's disease in people with 65 year of age is 13%, which is expected to reach 20% by 2030 (McDowell, 2001). The most common type of Alzheimer's disease is the late onset type which affects after the age of 60 years. The prevalence is 13% in the people with 65 year age, reaching almost 40% in the 90-94 year age group (McDowell, 2001; Castro, 2010). The prevalence of Alzheimer's increases by each decade after a person attains the age of 65.

There is a genetic link for early onset and late onset of the Alzheimer's disease (Tilvis *et al.,* 2004). Five percent of the people with Alzheimer's are reported to have early onset of the disease, which makes it a rare type of Alzheimer's disease and is linked to mutations in certain genes. The symptoms usually appear between the age of 30 years and 50 years. It is known to be caused by the mutations in three genes, which were inherited from the parents. Chromosome 12 is suspected to have the gene, which is susceptible to Alzheimer's disease (Rader, 1995; Scott, 2000). The mutation on the chromosome 21 that encodes the amyloid precursor protein (APP) is linked to Alzheimer's disease. This protein is the precursor for the Aβ protein, which is associated with 2% to 3% of the early onset familial form of Alzheimer's disease (Aisen, 1997). The presenilin 1 gene is linked to 70% to 80% of the early onset of the

familial form of the Alzheimer's disease and presenilin 2 gene contributes to 20% to 25% of the early onset familial form of Alzheimer's disease. These genes alter APP processing, which causes the increase in the production of Aβ resulting from its proteolytic cleavage (Gambert, 1997; Forsyth, 1998). The gene on the chromosome 19, which encodes for apolipoprotein E, which is involved in the metabolism of cholesterol, is also associated with the onset of the late onset forms of Alzheimer's disease that accounts for 50% of total Alzheimer's disease occurrence (Forsyth, 1998). There are several other suspected risk factors involved in the development of Alzheimer's disease (Ham, 1997).

Alzheimer's disease on a micro level is characterized by three distinguishing characters like extracellular Aβ plaques, intracellular NFTs and neuronal cell degeneration (Graff-Radford *et al*., 1997). Alzheimer's disease is associated with abnormal depositions such as neurofibrillary tangles and Aβ plaques which destroy healthy neurons (Gilnian, 1997) in human brain. It was in 1960 that the scientists discovered that there is a link between the number of plaques present in the brain and Alzheimer's disease pathology. These abnormalities were found in the brain areas, which control the memory. However, the pathophysiology of this disease is in its infancy. Aβ plaques and intracellular neurofibrillary tangles (NFTs) in the brain were observed to be the major outcome of Alzheimer's disease. Apart from Alzheimer's disease, NFTs and plaques do also occur with natural aging and other neurodegenerative disorders. The neuropathology of Alzheimer's disease is characterized by the excessive deposition of insoluble Aβ ($A\beta_{1-40}$ and $A\beta_{1-42}$) aggregates and neurofibrillary tangles composed of tau amyloid fibrils in the astrocyte cells of the brain (Anantharaman *et al.,* 2006; Bajda *et al*., 2011). The aggregates are commonly called as senile plaques and it has been observed that higher the concentration of Aβ *in vivo*, the higher is the probability that they would aggregate into insoluble plaques (Mc Carty, 2006; Whiteley, 2014). Aβ plaques are considered to play a central role in Alzheimer's disease with several pathophysiological processes known as amyloid cascade, which was proposed by John Hardy in 1991. Thus, discovery of each and every pathophysiological process disclosed new possibilities in the discovery of new therapeutic targets (Hamdy *et al*., 1990).

NFTs containing hyperphosphorylated tau protein are another hallmark of Alzheimer's disease. Several research works demonstrated that dying neuronal cells are filled with

this protein in the areas of brain, where memories are made or stored and subsequently spread to the other cells which are involved in remembering and reasoning (Raymond, 1994).

1.1.2 Treatment of Alzheimer's disease and features of interest:

There is no treatment available till date (Weiner *et al.,* 2013) for complete cure of Alzheimer's disease. There are few drugs available in the market for the treatment of Alzheimer's disease with limited benefits. They can delay the onset of neurodegeneration or address the symptoms associated with Alzheimer's disease, but not the disease *per se.* Alzheimer's disease is a multifactorial disease associated with several risk factors. Currently available drugs are targeting various factors, which are involved in the pathogenesis of Alzheimer's disease and one such factor is enzymes. In 1997, several acetyl cholinesterase inhibitors such as Donepezil, Galantamine and Rivastigmine were introduced into the market and prescribed as major drugs for symptomatic relief (Birks, 2006; Bajda *et al*., 2011) in Alzheimer's disease patients. Memantine, NMDA receptor antagonist, was another class of drugs approved for the treatment of Alzheimer's disease for moderate to severe Alzheimer's disease (Bajda *et al*., 2011; Salomone *et al*., 2012; Lobello *et al*., 2012). Remaining symptomatic drugs are based on neuronal nicotinic, muscarinic, Gamma-aminobutyric acid (GABA), serotoninergic, histamine H3 receptors, and others like phosphodiesterase and Peroxisome proliferator-activated receptor gamma (PPAR-γ) receptors (Bajda *et al*., 2011). However, these drugs provide symptomatic and a temporary benefit, without affecting the underlying mechanisms of pathogenicity (Salomone *et al*., 2012; Lobello *et al*., 2012). Despite their use in the treatment of Alzheimer's disease, most of these drugs exhibit side effects (Howes, 2014).

There are other classes of drugs still under development known as disease modifying drugs, those targets factors related to Aβ, tau proteins and neuroprotection. These drugs are shown to attenuate the progression of Alzheimer's disease, however developing these drugs against Aβ and tau proteins is one of the biggest pharmacological challenges (Salomone *et al*., 2012). Drugs developed till date include drugs for prevention of Aβ accumulation and aggregation, promotion of Aβ clearance, the reduction of Aβ production and targeting of tau phosphorylation and assembly (Bajda *et al*., 2011;

Salomone *et al*., 2012). Aβ is a protein produced by enzymatic cleavage of amyloid precursor protein (APP). The key enzymes responsible for processing of APP, which leads to the production of Aβ, are α, β, and γ-secretases (Bajda *et al*., 2011). Hence, these enzymes act as important target for the Alzheimers disease in the development of disease modifying drugs. In addition to these classes of drugs, several other categories of drugs under investigation includes, anti-inflammatory medications, estrogen, antioxidants, calcium channel blockers and cholesterol lowering drugs (Kawas, 2006). Anti-inflammatory drugs and antioxidants can mitigate the inflammatory consequences and oxidative brain damage by reactive oxygen species (ROS) which are thought to be other important factors in the manifestation of Alzheimer's disease (Claudia, 2006; Bajda *et al*., 2011; Jaturapatporn *et al*., 2012).

Among the above mentioned drug targets, important target for reducing Aβ formation is the BACE1. BACE1 is also named as Asp2 and memapsin-2, is an aspartyl protease belonging to the pepsin family (Zhang *et al.,* 2010; Bajda *et al*., 2011). A decade ago, BACE1 was identified and cloned for further analysis, which led to the realization that this enzyme could act as a potential therapeutic target in Alzheimer's disease treatment (Klaver *et al*., 2010; Luo and Yan, 2010). Though there are several compounds screened for BACE1 inhibition and even some of the compounds are in clinical trials (Ghezzi *et al*., 2013; Karran *et al.,* 2011), there is no single disease modifying compound succeeded in phase-III clinical trials (Ghezzi *et al*., 2013). This situation urges to find new and potent disease modifying drugs such as BACE1 inhibitors. Natural molecules are better alternative to the present day synthetic drugs due to the higher safety profile when compared to synthetic compounds (Harvey, 2008).

Natural molecules have higher levels of success due to their lower toxicity. However, there are lots of challenges needs to be addressed during the development of the natural molecule as a potential drug candidate. In the current study, several natural molecules were screened and further evaluated for their efficacy as a potential therapeutic intervention for Alzheimer's disease.

1.2. Research Question:

The current research work was focused on screening and evaluating molecules from natural sources for therapeutic application in Alzheimer's disease. There are also efforts

made to solve the problem associated with phytochemicals such as poor aqueous solubility and bioavailability, thus increasing the *in vivo* efficacy.

Based on the information available so far, currently there are no drugs available in the market for the complete cure of Alzheimer's disease. The marketed drugs can either prolong the onset of the disease or can decrease the symptoms associated with Alzheimer's disease. Although they are efficient in reducing the disease burden to some extent, there are side effects associated with these drugs. There are several drugs under development and are in different stages of drug discovery, but none of them has made it to the market so far. Several synthetic drugs failed during their development as a treatment due to either efficacy or toxicity. The current information on Alzheimer's disease treatment clearly points out that, there is a need for drugs which can cure the disease with no or minimal side effects.

The possible alternative solution to present day synthetic drugs would be screening natural compounds (phytochemicals) of potential anti-Alzheimer's disease activity. Natural molecules from plants and animal are used for human applications from time immemorial and are found to be safe for human consumptions. They are also found to be effective in the treatment of several diseases and also acted as a source of new drugs. In the current study, natural molecules were screened against a validated drug target and studied for their efficacy as a potential treatment for Alzheimer's disease.

1.3. Research Objectives and proposed methodology:

The present doctoral thesis work is mainly aimed at finding a potential drug candidate to treat Alzheimer's disease with low or no toxicity. The key objective was to screen natural molecules against one of the validated drug target, BACE1 and further study the efficacy of screened molecules in *in vitro* and *in vivo* studies.

To accomplish this objective, a systematic approach was executed starting from literature search for natural molecules with potential neuroprotective activity, screening for natural compounds in *in silico* and *in vitro* studies and then evaluate efficacy in *in vivo* animal study for validation of potential compounds. Appropriate drug target was selected from the available scientific literatures for screening the compounds. The drug target selected for screening and evaluation of potential drug candidate is Beta site APP cleaving enzyme-1 (BACE1), also called β-secretase. Short-listing of potential natural

compounds of plant and animal origin was done through literature search, in relation to plants used in traditional treatment of neurological disorder. Short-listed compounds were then analyzed using *in silico* tools for binding efficacy against BACE1 through molecular docking studies. Subsequently, *in vitro* and *in vivo* experimental validations of selected natural compounds were performed.

The systematic objectives for the present study are as follows:

1. Literature search and selection of natural compounds with potential neurological applications.
2. Screening short-listed compounds using *in silico* docking against BACE1.
    a. Molecular docking study using AutoDock 4.0.
    b. Drug likeliness analysis using MolSoft and Osiris property explorer.
3. *In vitro* evaluation of short-listed compounds from *in silico* studies:
    a. Measuring the free radical scavenging activity using ABTS (2, 2-azinobis (3-ethylbenzothiazoline-6-suifonic acid)) radical cation decolorization assay.
    b. Measuring the free radical scavenging activity using DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method.
    c. Measuring the free radical scavenging activity using Ferric Reducing Antioxidant power (FRAP) assay.
    d. Measuring the free radical scavenging activity using superoxide scavenging assay.
    e. Evaluating Lipoxygenase (LOX) inhibition activity of the selected compounds by LOX inhibition assay.
    f. Evaluating BACE1 inhibition activity of selected compounds using FRET assay.
4. Formulation development for improving the aqueous solubility of shor-tlisted compound.
5. Evaluation of efficacy of shor-tlisted compound *in in vivo* animal model study using aluminium chloride-induced Alzheimer's disease animal model.

1.4. Research Contribution:

The current research work mainly concentrated on evaluating natural compounds for potential therapeutic application in the treatment of Alzheimer's disease. Since the natural molecules are safe for human consumption, considering them for the development of drug reduces the failure rate of drugs in later stages of drug discovery and development. Research work proposed a lead molecule, which can be further tested in human clinical studies for safety and efficacy. In this research work, *in silico* tools were used for the initial screening natural molecules, which reduces the time and cost of drug development.

In conclusion, the research work proposed a potential multi target drug candidate for therapeutic application in the treatment of Alzheimer's disease, which can be further developed into a drug as per the regulatory requirement and commercialized.

1.5. Thesis Outline:

The present thesis is structured as below:

Chapter-1: Introduction

This section introduces the research topic, research problems, research objectives and methodologies undertaken while carrying out the research work. This section discusses important aspects in understanding Alzheimer's disease and its pathophysiology, etiology, and treatment with recent updates. It also discusses the problems associated with currently marketed drugs and future needs. This section gives introduction about the Alzheimer's disease starting from a brief history till the research problem considered for this thesis work. The contributions of the research work, future studies and thesis outlines have also been briefed.

Chapter-2: Literature review

This section discusses in detail about the previous literatures available which are relevant to Alzheimer's disease. It discusses about disease pathophysiology, possible causes of the disease, case studies, present detailed understanding of Alzheimer's disease and its drug target, advances in diagnosis, possible preventive measures,

available treatment methods, advantages and disadvantages of the presently available drugs.

Research Contribution:

The research contribution section of the thesis discusses the research work carried out in details with methodologies adopted. The research contribution section is further divided into five different chapters, viz Chapter-3, Chapter-4, Chapter-5, Chapter-6 and Chapter-7. Each of the chapters has specific and short literature review in their introduction section to provide more clarity and a view of the earlier work relevant to each research chapter.

Chapter 3:

This chapter introduces the natural compounds (Phytochemicals) with potential neurological applications. The natural compounds were short-listed based on the evidence of application in neurological disease from earlier research. The short-listed phytochemicals from this chapter were considered for *in silico* screening in Chapter -4

Chapter 4:

This chapter introduces the *in silico* tools considered for the research work, screening methodologies and *in silico* screening results. In this chapter methodologies and results of *in silico* screening studies carried out to determine drug like properties of screened natural compounds are also discussed. This section summarizes the *in silico* work carried out and suggests the natural compounds which can be studied further in *in vitro* tests in Chapter-5

Chapter 5:

This chapter introduces the different *in vitro* tests carried out on short-listed compounds (two compounds) from *in silico* studies and their results. The materials and the methods used for the *in vitro* tests were discussed in details with the results. This chapter reconfirms the results obtained in the *in silico* studies and proposes compounds for formulation development and further evaluation in *in vivo* animal model study.

Chapter 6:

This chapter introduces the formulation development done for short-listed compound to increase its aqueous solubility. The formulated compound was further considered for evaluating its efficacy in *in vivo* animal model study.

Chapter 7:

This chapter introduces the animal model used for evaluating the short-listed compound, animal model development, experimental methods carried out, and the results. The work under this chapter confirms the efficacy and safety of the short-listed compound in *in vivo*.

Chapter-8: Discussion and Conclusions:

This section discusses the outcomes of Chapters 3, 4, 5, 6 and 7 in comparison with existing research findings available from the literature and also the conclusion. The overall contribution from the current research is highlighted and required further research is briefed.

Chapter-9: Future work:

This section proposes future research required for developing the screened and evaluated compound for the potential therapeutic application in the treatment for Alzheimer's disease.

Chapter 10: References:

This chapter lists all the references used in this thesis.

Chapter 11: List of Appendices:

This chapter lists all the appendices to this thesis.

Chapter-2

2. Literature Review

2.1 Overview of Alzheimer's disease:

Alzheimer's disease, the most common form of dementia, is an irreversible, progressive neurodegenerative disease characterized by cognitive, behavioral and functional impairment (Castro *et al.,* 2010; Baquero and Martin, 2015). The changes in the functioning of the brain results in the emotional and neurocognitive reduction, which increases the reliance on others and decreases in the functional mobility, thereby the quality of life (Castro *et al.,* 2010; Aisen, 2011). Alzheimer's disease is prevalent in elderly persons, but also can affect the younger people in smaller portions. The brain damage begins in the least myelinated areas of the brain, more specifically in the hippocampus, which results in the loss of recent memories. The neuronal degeneration starts from the hippocampus and subsequently spread to the frontal cortex, which affects the planning ability and concentration. There is a global pandemic involving millions of victims of Alzheimer's disease (Foster, 2004; Akbaraly *et al*., 2009). The rate of the population being affected by Alzheimer's is rising significantly than the aging population (Brayne *et al*., 1995). According to Alzheimer's disease statistics in 2015 and World Alzheimers Report, 2015, globally nearly 44 million people have been found to have Alzheimer's disease or a related dementia and are expected to increase to 131.5 million by 2050 (Prince *et al.,* 2015).

In 1901, the first patient named, Auguste Deter with Alzheimer's disease was reported for being mentally ill and epileptic in the hospital at Frankfurt. Auguste Deter was only 51 years of age, when she developed the symptoms of Alzheimer's disease (Graeber *et al.,* 1998; Graeber *et al.,* 1997). In 1996, Alzheimer's disease was noted to be the cause of death for around 21,397 people. In 1999, the death caused by Alzheimer's rose to 44,509 persons in the USA alone (Foster, 2004). Globally, it is estimated that, Alzheimer's disease affects 10% of the population over the age of 65 (Zhang *et al.,* 2011).

Alzheimer's disease is mainly dependent on factors related to aging and familial background with the prevalence rate of 90% and 10%, respectively, among Alzheimer's disease patients (Bekris *et al.,* 2010). Age related physiological changes have been strongly correlated to Alzheimer's disease. Apart from aging, several other risk factors are also involved such as stress, inflammation, head injury and intake of neurotoxic substances unknowingly along with food (Holcomb *et al.,* 1998; Rubio-Perez, 2012). So far, several genes have been identified as risk factors, which are very important in the inheritance of familial Alzheimer's disease. Certain mutations in specific genes of ancestors become more strongly influential and make the next generations to be vulnerable to Alzheimer's disease. There are four genes identified till date, which play important roles in early onset and late onset of Alzheimer's disease; these genes code for apolipoprotein E, amyloid precursor protein (APP), presenilin 1 (PS_1) and presenilin 2 (PS_2) (Holcomb *et al.,* 1998; Saraceno *et al.,* 2013). Mutations in APP, PS_1 and PS_2 genes were correlated to early onset of the disease and accounts for 5% of total Alzheimer's disease. The mutations in the presenilin genes have been linked to more than 40 % of the familial cases of the Alzheimer's disease. Mutations in the gene coding for PS_1 result in the increased Aβ production, which leads to the Aβ peptide toxicity (Black, 1990; Newman *et al.,* 2007) due to its accumulation and subsequently neuronal death. APO E4 allele plays a key role in progression of Alzheimer's disease. Mutation in apolipoprotein E4 (APO E4) allele is strongly associated with late onset of disease and was known to increase disease risk by 12 folds (Bachman *et al*., 1993; Andersen *et al*., 1999; Emilien *et al.,* 2000; Selkoe, 2001; Liu *et al.,* 2013; Saraceno *et al.,* 2013).

Factors that are involved in clinical investigation of Alzheimer's disease include medical history and physical examination to identify different mood variations, behavioral changes and mood performance (Lemere *et al.,* 2010). Aβ plaques and NFTs are the key biomarkers for identifying Alzheimer's disease and are considered as possible therapeutic targets in drug discovery and development for the treatment of Alzheimer's disease (Lemere *et al.,* 2010). Intracellular neurofibrillary tangles (NFTs) and Aβ plaques are the hallmark of Alzheimer's disease brain pathology. NFTs are paired helical filaments of aberrantly hyperphosphorylated Tau (Rudrabhatla, 2011; Alzheimer's disease progress report, 2011-2012) and Aβ plaques are aggregates of Aβ proteins.

During 1984, Glenner and Wong identified amino acid sequence of Aβ and then named it as Aβ protein (Tanzi *et al.,* 2005). It is produced by the enzymatic cleavage of a transmembrane protein called Amyloid Precursor Protein (APP) (Gabelle *et al.,* 2010). APP is processed by three different enzymes called α secretase, β secretase (BACE1) and γ secretase into small fragments in the brain. In Alzheimer's disease brain, APP is enzymatically cleaved by BACE1 and subsequently by γ secretase into Aβ peptides. These excess Aβ peptides get accumulated as insoluble plaques called Aβ plaques forming gum-like deposition (Foster, 2004). Excessive production of Aβ and its subsequent accumulation as Aβ plaques results in pathological events such as synaptic loss, and loss of neurons (Zhang *et al.,* 2011). These Aβ plaques and NFTs together damage the synapses of the neurons, which in turn interfere with the communication of neurons (Fratiglioni *et al*., 2004; Zuo *et al.,* 2015) affecting memory. Further to the formation of amyloid plaques, free radicals of oxygen are formed that are highly reactive and causes oxidative stress in the brain (Ahsan *et al.,* 2003), finally, leading to the inflammation and death of the neuronal cells.

Tau is a protein which helps in stabilization of neuronal microtubule and also in the transportation of the nutrients and other molecules in the brain via microtubules (Copeland *et al*., 1999). In Alzheimer's patients, the tau becomes hyper phosphorylated and tangled with other tau proteins, forming NFTs leading to the disintegration of the microtubules. Disintegration of microtubules affects the intraneuronal communications and nutrient transport. These NFTs restrict the movement of molecules and nutrients to the end of the neurons thereby restricting the transportation of nutrients in the neuronal cells. These NFTs interfere with the function of the temporal lobes, which causes writing difficulties and memory loss. When the NFTs start accumulating in the frontal lobes, other disorders like personality disorders start appearing (Galasko *et al*., 1994).

In addition to this, the NFTs and Aβ plaques damages the grey matter leading to progressive cerebral atrophy and loss of cognitive functions (Foster, 2004; Carrasquillo *et al*., 2010).

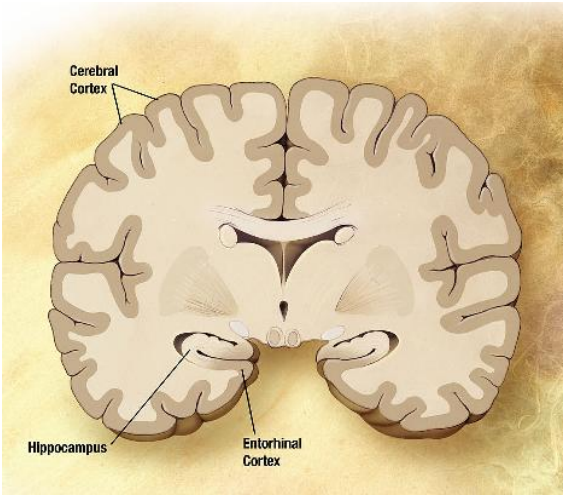

Figure 1. Normal brain having healthy hippocampus and cortex regions (Source: Chris, 2013).

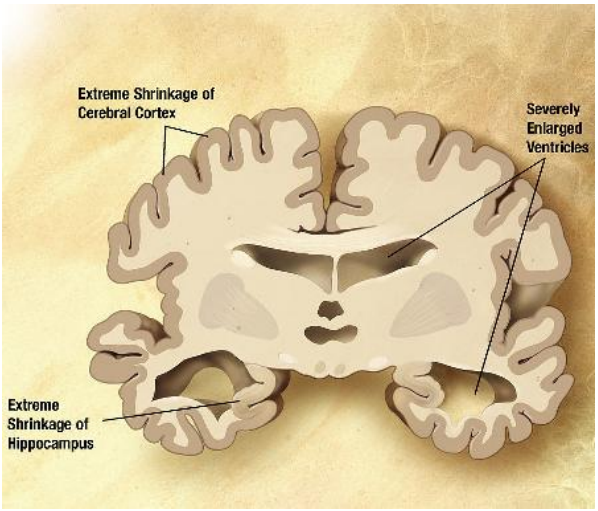

Figure 2. Alzheimer's diseased brain having abnormal hippocampus, cortex and ventricle regions (Source: Chris, 2013).

2.2 History and Epidemiology:

Alzheimer's disease was first discovered in a person called Auguste Deter by Dr. Alois Alzheimer in 1907. Autopsy of the Auguste Deter's brain after her death showed neurons with unusual depositions known as tangles and senile plaques. It was hypothesized by Dr. Alzheimer that the lesions in the brain are responsible for the memory loss. Later, several people were reported to have the similar symptoms which led to this disorder to be called as Alzheimer's disease (Poirier *et al*., 2001). Earlier, the symptoms were considered as natural phenomenon due to progression of age and latter found that, Alzheimer's disease is closely linked to ageing (Castro, 2010). In addition to aging, environmental and genetic factors play a major role in Alzheimer's disease (Deason *et al*., 2013). There have been a several gene mutations and proteins were found to be the cause of Alzheimer's disease, but it was not known how the genetic mutations work in the onset of the disease. The factors which contribute to Alzheimer's are not completely known yet.

Alzheimer's disease is categorized into mild, moderate and sever based on the severity of the disease symptoms. Mild type of Alzheimer's disease is more prominent than others, such as moderate and severe (Reitz *et al*., 2011). The person identified with Alzheimer's disease can survive up to 3 to 10 years and the survival time depends on the age at which the disease is diagnosed. It was reported that the Alzheimer's disease is the eight leading cause of death with a progressing age (Verghese *et al*., 2003; Castro, 2010). Globally, it is estimated that, Alzheimer's disease affects 10% of the population over the age of 65 (Zhang *et al.,* 2011).

The major milestones in Alzheimer's disease research are listed below in chronological order (Source; http://www.alz.org/research/science/major milestones in alzheimers.asp, viewed on 1st Feb, 2016).

1906 : Alzheimer's disease was first discovered by Dr. Alois Alzheimer, a German psychiatrist and neuropathologic, in a patient named Auguste Deter with memory loss.

1910 : The disease was named as Alzheimer's disease by Emil Kraepelin in the name of Dr. Alois Alzheimer.

1968 : A validated cognitive measurement scale was developed to assess cognitive and functional decline.

1976 : Alzheimer disease was found to be the most common cause of dementia by a neurologist called Robert Katchman.

1984 : First time Aβ was identified, named and its amino acid sequencing was carried out by Glenner and Wong.

1986 : Tau protein was discovered as the second pathological hallmark Alzheimer's disease.

1987 : Clinical trial with Tacrine was initiated.

1987 : The first gene on chromosome 21 associated with Alzheimer's disease was identified.

1993 : Apolipoprotein E4 (APO E4) was identified as risk for Alzheimer's disease.

1993 : Tacrine was approved by USFDA for the treatment of Alzheimer's disease.

1995 : First transgenic (Tg) mouse model was developed by cloning human APP gene.

199 Vaccines were tested on transgenic mice model.

2004 : PET imaging was used for detecting Alzheimer's disease.

2010 : Health statistic report of US by CDC showed that Alzheimer's disease is 6th leading cause of death in the US.

2.3 The economic effect of Alzheimer's disease:

The social, economic and health care burden due to Alzheimer's disease is very high (Castro, 2010) which creates enormous strain on the global health care system. Alzheimer's disease comes under the broad neurological disorder called dementia. However, Alzheimer's disease occupies 60-70% of total dementia (WHO, 2015). Globally, the total number of dementia patients, in 2010 was estimated to be 35.6 million and is estimated to reach 115.4 million by 2050 (WHO, 2013). In USA, Alzheimer's disease has affected more than 5 million people among which 0.2 million people are under the age of 65 years (Morris, 1994). The death rate due to Alzheimer's disease increased to 68% where as it was significantly decreased for major diseases like

HIV, stroke and heart disease. Alzheimer's disease is associated with high cost of treatment and care due to cognitive impairment in Alzheimer's patient leading to impaired in financial and cognitive skills (Snowdon, 2002). The patients with Alzheimer's disease have a high risk of losing their acquired qualities and financial skills, which causes decline and eventual loss of productivity (Chen, 1998). Handling financial tasks become extremely problematic for the patients without assistance from caregivers. It was identified that there is a decline in financial skills in patients with mild cognitive impairment before the development of Alzheimer's disease. Compared to cancer and coronary heart diseases, Alzheimer's is third in place for its treatment cost, in the United States (Hendrie, 1998). The cost of treatment and care taking per year for dementia patients including Alzheimer's diseased patients is estimated to be more than USD$ 604 billion (WHO, 2013). In this, the average cost of treatment per person for Alzheimer's disease is three times more than the other dementias (Geldmacher *et al*., 1996) and is due to multiple medical conditions in Alzheimer's diseased patient. Also, the caretakers of diseased patients experience a high level of emotional stress. The factors considered for cost of Alzheimer's disease were cost of medications, care taker cost and loss of productivity. The indirect costs of disease are more than direct costs (Shah, 2000). The costs are distributed accordingly, into 60% for indirect cost and 40% for direct cost. Due to the nature of disease, progressive impairment, functional status and behavioral aspects, burden on the patient care increases significantly. The number of hours, which are required for caretaking increased from 11 to 70 hours a week (Evans *et al.,* 1990; Castro, 2010). The direct cost of Alzheimer's disease treatment is from medications which are expensive, but delay in the progression of the disease due to the medication reduces the direct costs in later period (Honig *et al.,* 2001; Castro, 2010).

2.4 Predisposing factors:

Alzheimer's disease is a multifactorial disease, in which various factors are involved in the onset of the disease. The major factors involved are age, environment, genetics and lifestyle factors (Knopman *et al*., 2010). These factors are different from each other and hence, controlling all of them at a time is difficult. Advancing aging is one of the highly correlated factors in the development of Alzheimer's disease and is known to develop after the age of 65 years (Skoog *et al.,* 2003; Castro, 2010). The overall percentage of Alzheimer's disease occurrence in 65 year of age is 13%, which is expected to reach 20% by 2030 (McDowell, 2001). The disease prevalence rate is almost 40% in the 90 to 94 year age group (Castro, 2010). Although Alzheimer's disease was considered to be occurring in elderly after the age of 65 years which is called as late onset Alzheimer's disease, five percent can occur in the people with age of below 65 years, which is called as early onset Alzheimer's disease. Scientists have found out that there is a genetic link for early onset and late onset of the Alzheimer's disease (Tilvis *et al.,* 2004). The symptoms usually appear between the age of 30 years and 50 years. It is known to be caused by the mutations in three genes, which were inherited from the parents. The most common type of Alzheimer's is the late onset type which affects at the age of 65 years.

Apolipoprotein E (APOE), a major cholesterol carrier is one of the several risk factors. It has three alleles (Flicker, 2010) in which, APOE E4 increases the risk of occurrence of Alzheimer's disease. Estrogen hormone is linked to Alzheimer's disease thus making females more susceptible to Alzheimer's disease than men (Kamat, 2010; Figure 3). The reduced level of insulin in the brain in the conditions such as diabetes is known to be a risk factor (Rockwood, 1997). Down-syndrome and psychosocial stress are also considered as important risk factor for developing Alzheimer's disease. It was reported that negative events in life can be a reason for developing Alzheimer's disease. Association of other risk factor such as oxidative stress and inflammation in the brain are also reported in the literature. Some of the metals ions are known to increase the risk of Alzheimer's disease and research is still going on to know the effect of aluminum, copper and iron in the development of Alzheimer's disease (Licastro *et al*., 2000). Aluminium, a known neurotoxin is highly correlated with Alzheimer's disease. The disease risk is coherently increasing and furthered by the canned foods in aluminium

containing packing materials (Klimkowicz *et al*., 2002). However, aluminum which was considered as a risk factor is no longer that much considered (Licastro *et al*., 2000).

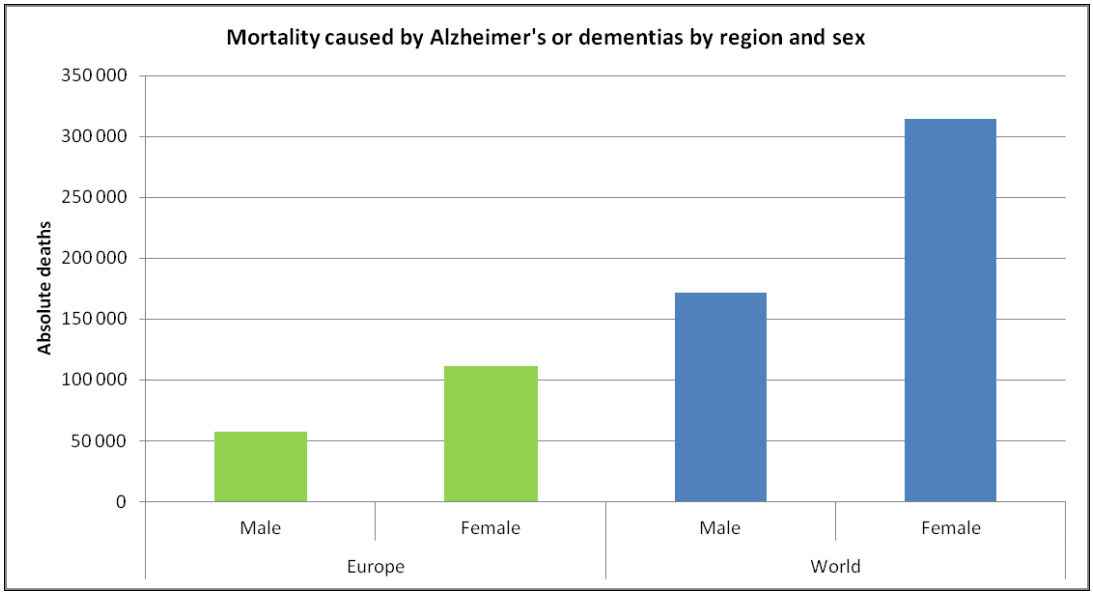

Figure 3. Gender based absolute death by Alzheimer's disease in Europe and the world (Graph obtained from World Health Organization., 2013).

2.5 Pathophysiology of Alzheimer's disease:

The mechanism underlying Alzheimer's disease is not clearly understood so far. However, it is widely accepted that genetic factors and environmental factors are responsible for the onset of Alzheimer's disease (McGleenon *et al.,* 1999). Alzheimer's disease is multifactorial in nature and has several hypotheses linked to its pathophysiology. The major pathophysiology of Alzheimer's disease includes the plaques formed by Aβ accumulation and aggregation of hyperphosphorylated tau protein into NFTs (Hardy, 2002; Bajda *et al*., 2011) and neuronal cell death (Morrison *et al.,* 2005). Aβ is a protein produced by enzymatic cleavage of amyloid precursor protein (APP). Amyloid precursor protein (APP) is a transmembrane protein, which is normally expressed in the CNS and brain (Resende *et al*., 2008). The key enzymes responsible for processing of APP are proteases called α secretase, BACE1, and γ-secretases (Bajda *et al*., 2011). Enzymatic cleavage of APP by BACE1 followed by γ-

secretases leads to the production of Aβ and is called as amyloidogenic pathway (Eckerta *et al*., 2008) which is one of the well studied pathways for its role in Alzheimer's disease (Morrison *et al.,* 2005). Aβ has good affinity between each other and can bind to each other to form oligomeres (Aβ plaques) which can bind to neurons leading to the dysfunction of the neuronal network in the Alzheimer's disease brain (Um *et al*., 2012). The mutation in the APP gene favors amyloidogenic pathways and causes early onset of the Alzheimer's disease in the population of below 65 years of age (Nimmrich *et al*., 2008).

Tau is an important protein involved in the assembly and stability of microtubule in neurons. Its hyperphosphorylation leads to its detachment from the microtubule and its intracellular aggregation called NFT. Hyperphosphorylation of tau protein and subsequent aggregation leads to destabilization of microtubule (Iqbal, 2004) causing neuronal cell death. Microtubules are involved in neurotransmission and communication which gets lost due to the hyperphosphorylation of tau protein and its subsequent accumulation as NFTs (Morrison *et al.,* 2005). Accumulation of both extracellular Aβ and intracellular NFTs ultimately leads to neuronal cell death and subsequently, cognitive decline.

Though there were several research studies focused on possible cause of the Alzheimer's disease onset, there is no confirmatory or single cause found to act exclusively for the disease. However, there are many hypotheses tested based on many clinical observations and other scientific analysis, which mainly revolves around cholinergic hypothesis, amyloid cascade hypothesis and tauopathies. The following section discusses the details of such hypothesis and possible evidences to prove the same.

2.5.1 Proposed Alzheimer's disease hypothesis:

2.5.1.1 Cholinergic hypothesis:

Cholinergic hypothesis is one of the important and early hypotheses in the research on Alzheimer's disease pathophysiology. Sims and his coworkers (Sims *et al.,* 1981) first proposed cholinergic hypothesis for Alzheimer's disease pathophysiology. According to this hypothesis, the synthesis of neurotransmitter called as acetylcholine is low in the

brain of an Alzheimer's disease patient (Guidi *et al*., 2006). Acetylcholine, a neurotransmitter plays very important role in memory formation and recall. The activity of choline acetyltransferase found to be decreased in Alzheimer's diseased brain affecting cholinergic neurons ultimately, leading to memory loss. There was more than 75% of loss in cholinergic neurons reported in the literature (Morrison *et al.,* 2005). Several drugs were developed based on this hypothesis for the treatment of Alzheimer's disease (Perry *et al.,* 2008) which includes cholinergic neuron agonists, acetylcholinesterase inhibitors and acetylcholine releasing agents. Among these, inhibition of acetylcholinesterase was highly explored which led to the development of three drugs such as galantamine, rivastigmine and donepezil, which are approved for the treatment of Alzheimer's disease (McGleenon *et al.,* 1999; Patwardhan *et al*., 2009). However, these drugs gives relief from the symptoms associated with Alzheimer's disease but do not treat the cause. Research is on for developing efficient drug delivery system, so that the drug molecules can pass through the Blood Brain Barrier (BBB) (Cedergren *et al*., 2007).

2.5.1.2 Hypothesis of oxidative stress and imbalance:

According to the recent studies, oxidative stress is considered as one of the risk factor in the pathophysiology of Alzheimer's disease. Most research studies showed good correlation between the oxidative stress and Alzheimer's disease (Gracy *et al*., 1999). The reactive oxygen species (ROS) are the main causative factor for oxidative stress and higher production of ROS is involved in pathogenesis of Alzheimer's disease. Higher amount of ROS in the brain can damage DNA, leads to lipid oxidation and protein oxidation, ultimately leading to tissue damage (McCann *et al*., 2005; Feng *et al.,* 2012). Oxidative imbalance in the brain causes neuronal cell death leading to pathogenesis of Alzheimer's disease. Several natural and synthetic antioxidants were explored for their therapeutic application in Alzheimer's disease with inconclusive results (Mancuso *et al.,* 2007).

2.5.1.3 Nitric oxide hypothesis:

Nitric oxide (NO) and reactive nitrogen are involved in physiological events like neuromodulation and neurotransmission (Aliyev *et al*., 2004). These are

thermodynamically very unstable resulting in cross reaction causing nitrosylation and nitration of proteins. Nitric oxide is involved in the pathogenesis of Alzheimer's disease in multiple ways such as activation of inflammatory process and oxidative stress ultimately leading to neurodegeneration. It is identified that, nitric oxide synthetase (NOS) are highly expressed in Alzheimer's disease brain and they cause higher level of nitric oxide leading to neuronal cell degeneration (Mohandas *et al.,* 2009). The oxidative stress caused by elevated levels of nitric oxide leads to neuronal cell apoptosis by initiating redox reactions, which subsequently affects the cognitive functions (Calabress *et al*., 2000; An *et al.,* 2008). Also, nitric oxide was known to cause hyperphosphorylation of tau proteins (An *et al.,* 2008), which is one of the hallmark in the pathogenesis of Alzheimer's disease.

2.5.1.4 Calcium hypothesis:

The levels of calcium Ca^{2+} in the brain is known play an important role in the pathogenesis of Alzheimer's disease. Also, its association with Alzheimer's disease is well established through several human and animal studies (Khachaturian, 1989; Mattson and Chan, 2001). It is one of the important metal ions involved in brain development (Mattson and Chan, 2001). It is suggested that the disturbance in homeostasis of calcium ions in the brain is responsible for neurodegeneration during Alzheimer's disease. The infiltration of calcium ions into the endoplasmic reticulum (ER) due to accumulation of Aβ enhances the sensitivity of the ryanodine receptors (RYRs) which increases the outflow of Ca^{2+} from the internal stores (Berridge, 2010). It is assumed that such dramatic changes in Ca^{2+} ions may result in the learning and memory difficulties during the onset of Alzheimer's disease (Danysz *et al.,* 2012). Aβ is known to affect calcium ion homeostasis through oxidative stress which leads to neuronal cell death, ultimately affecting the cognitive functions (Mattson and Chan, 2001).

2.5.1.5 Hypothesis of microtubule instability:

Microtubules are key components of the cytoskeleton of neuronal cells. They are filamentous, long, tube-shaped protein polymers, which are essential for intracellular transport and cellular metabolism (Andreadis *et al*., 1992; Jordan and Wilson, 2004;

Parker *et al.,* 2007). They are crucial in the maintenance and development of cell shape, cell signaling, intracellular transport and cell division (Goedert *et al*., 1988). Microtubule is heterodimeric in nature consisting of α and β-tubulins, which are arranged in the form of slender filamentous tubes, which can be many micrometers long. They are highly dynamic polymers α and β-tubulins and their dynamics in living cells are tightly regulated both temporally and spatially (Lindwall *et al*., 1984; Jordan and Wilson, 2004; Jain *et al,* 2013). The functional diversity of microtubules is achieved through the binding of various regulatory proteins through several post-translational modifications of tubulin (Biernat *et al.,* 1993; Jordan and Wilson, 2004). Tau protein hyperphosphorylation on neuronal microtubule causes disruption of microtubules, which results in axonal and dendritic degeneration during the onset of Alzheimer's disease. A lot of studies have shown that there is a decrease in tau phosphatase activity (PP2A) due to the hyperphosphorylation of Tau (de La Monte *et al*., 2003) The rationale behind microtubule binding drug approaches in tauopathies is to stabilize the microtubules by reducing hyperphosphorylation of tau thereby preventing the formation of NFTs (Kowall *et al*., 1987; Caceres *et al*., 1990).

2.5.1.6 Beta amyloid hypothesis:

Amyloid cascade hypothesis proposes that the Aβ production, oligomerization and its plaque formation causes inflammation, neurotransmitter deficits, neuronal dysfunction, neuronal cell death and ultimately, loss of cognitive functions (Phiel *et al*., 2003). Amyloid cascade hypothesis is very well studied and recognized in pharmaceutical research (Solano *et al*., 2000), and is supported by several research studies. Aβ is a protein produced by enzymatic cleavage of amyloid precursor protein (APP) on neuronal cell membrane in the brain (Resende *et al*., 2008). The key enzymes responsible for processing of amyloid precursor protein are α secretase, BACE1, and γ secretases (Bajda *et al*., 2011). Enzymatic cleavage of APP by BACE1 followed by γ secretase leads to the production of Aβ proteins and this pathway is called as amyloidogenic pathway (Eckerta *et al*., 2008). Amyloidogenic pathway plays a central role pathogenesis of Alzheimer's disease (Morrison *et al.,* 2005; Bhaskar *et al.,* 2012). BACE1 is the first enzyme initiating the amyloid cascade to produce Aβ and hence, it is considered as a rate limiting step in the formation of Aβ. It is one of the important therapeutic targets in the development of drug for the treatment of Alzheimer's disease

(Chami *et al.,* 2012). There are commonly 40 amino acid residues in Aβ protein, but 5% of the proteins are known to contain 42 residues (Johnston *et al*., 2003).

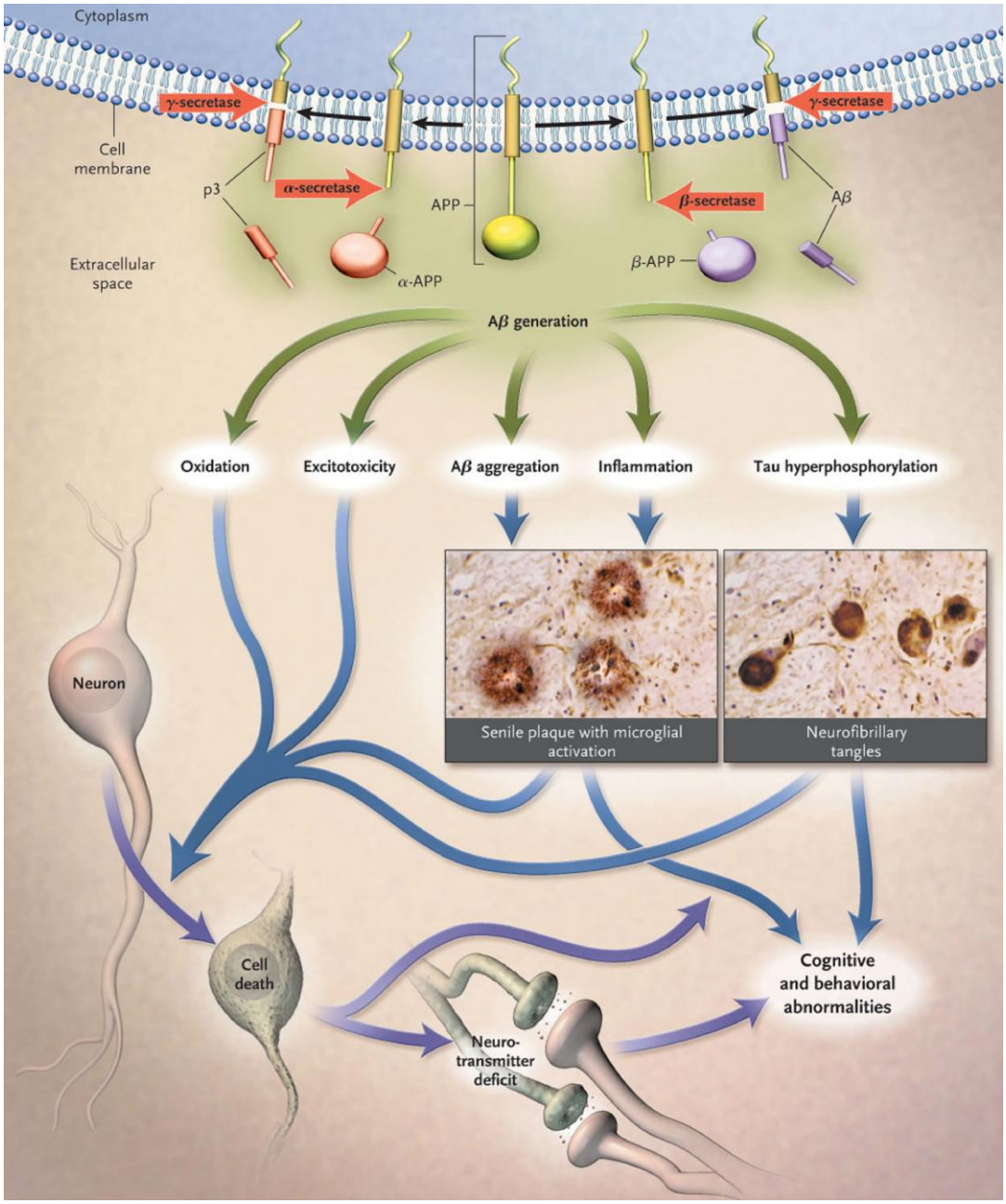

Figure.4. Amyloid cascade in Alzheimer's disease (Source: Ghareeb *et al.,* 2013)

The formation of more number of Aβ plaques can be due to the mutations in three genes such as PS I, PS II, APP, which are responsible for familial type of Alzheimers disease (Cross *et al*., 1995). Many transgenic mouse models have been designed to link between number of mutations and the Alzheimers disease (Ishiguro *et al*., 1993). The first transgenic mice was developed representing early onset of familial type Alzheimers

disease by the expression of human APP gene (Monte *et al*., 2006). APP is a direct precursor for the amyloid peptides and the mutations causes the overproduction of Aβ peptides. The developed transgenic mice produced the signs of synaptic loss, dystrophic neuritis, gliosos, accumulation of tau and NFTs (Pei *et al*., 1999). Overproduction of Aβ in transgenic mice, in particular, can be improved by introducing multiple transgenic lines. Mice with double transgenic qualities, which co-express both APP and Presenilin causing early onset of the Alzheimer's (Ho *et al*., 2004) are developed. The mice, which were triple transgenic co-expressing PS1, APP and tau mutations, demonstrated development of plaque in 6 months and tau expression at 12 months (Velliquette *et al*., 2005). Transgenic animal models are highly used for development of therapeutic interventions for Alzheimer's disease, more specifically for evaluating the efficacy of BACE1 inhibitors. Even though the amyloid cascade hypothesis based drugs have reached phase III clinical trials, none have made it into the market (Ishiguro *et al*., 1993).

2.6 Diagnosis:

Diagnosis and treatment of Alzheimer's disease is very important to reduce the disease burden. There is no single, validated evaluation method for proper diagnosis of Alzheimer's disease, since the patient suffers from memory loss. It is very important to have accurate clinical diagnosis of Alzheimer's disease even to carry out clinical trials with new therapeutic interventions (Daffner, 2000). Earlier, postmortem was considered as the only way to definitive diagnosis of Alzheimer's disease. The clinical diagnosis can give information on probable Alzheimer's disease. Generally, it is possible to diagnose Alzheimer's disease only when it is severe (Holmes, 2014). A group from the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's disease and Related Disorders Association (ADRDA) established Alzheimer's disease diagnosis criteria called NINCDS-ADRDA. This criterion addressed disease diagnostic problems associated with medical history, neuropsychological testing, laboratory assessment and clinical examinations (Moss, 1984; McKhann *et al.,* 2011) with 81% sensitivity and 71% specificity. They were also considered as reliable diagnostic criteria for possible Alzheimer's disease and were referred from 1984 till 2011 before its revision. After 27 years of usage, NINCDS and the Alzheimer's Association revised the criteria in 2011 for Alzheimer's disease

diagnosis. Both of these criterias required declining memory and other cognitive functions in the patients and now these criterias are used as guidelines by the clinical practitioners (Daffner, 2000).

Detailed family history and medical history of the patient is very important in the diagnosis of Alzheimer's disease and may require the help from caretaker. Mental state examination helps in understanding the stage of cognitive decline and can be done using standard tools such as Mini Mental State Exam (MMSE) and the Blessed Dementia Scale (Daffner, 2000; LaBar *et al.,* 2000). MMSE is an evaluation tool containing 11 questions that measures orientation, registration, attention and calculation, recall, and language representing cognitive functions (Miller *et al.,* 2012). The MMSE evaluation is a quick and validated tool which takes about 5 to 10 minutes for the evaluation and can clearly identify cognitive decline in the patients. Although it is effective, it has a drawback of completely relying on verbal communication and writing, which is very difficult for patient with speech and hearing problem. This also depends on literacy of the patient (Kurlowicz and Wallace, 1999), which is another drawback of this tool.

The structural abnormalities responsible for cognitive decline can be identified using neuroimaging such as computed tomography and magnetic resonance imaging (MRI) (Daffner, 2000). Neuroimaging can identify biological markers that include brain atrophy by MRI, reduced glucose metabolism in the brain regions with 18F-fluorodeoxyglucose-positron emission tomography (FDG-PET) and cerebral blood flow imaging with single photon emission computed tomography (SPECT) (Park *et al.,* 2014). Aβ protein imaging in the brain using positron emission tomography (PET) can be a very sensitive tool in diagnosing Alzheimer's disease. White matter disruption in the brain during Alzheimer's disease pathology can be identified by MRI (Ferreira and Busatto, 2011). However, the findings from neuroimaging are not specific to Alzheimer's disease.

2.7 Drug Targets:

2.7.1 Current Drug Targets:

Alzheimer's disease is a heterogeneous disease with several causative factors for which there is no drug available for the proper treatment or cure. Currently, the available drugs can either delay the onset of the disease or are symptomatic in effect. There were several drug targets identified and tested in the drug development for the treatment of Alzheimer's disease. APP, Aβ, BACE1, presenilins (PS1, PS2), γ-secretase, tau proteins, cholinesterase and several genes such as apolipoprotein E (APOE), were explored as drug targets in the development of drug for Alzheimer's disease. There are several acetylcholinesterase (ChE) inhibitors developed and are available for the treatment of Alzheimer's disease (Jagust, 2001; Lahiri *et al.,* 2002) but are symptomatic in effect. Many of the CHEIs, apart from inhibition of acetylcholinesterase enzyme, were shown to reduce levels of APP and Aβ via suppression of APP translation (Yogev-Falach, 2006), which is another novel property of certain cholinesterase inhibitors. Treatment of a human neuroblastoma cell line with CHEIs such as tacrine or phenserine significantly reduced the levels of Aβ (Cherny *et al*., 2001; Lahiri *et al.,* 2002; Yogev-Falach, 2006). The effect of CHEIs on APP levels is different from that of muscarinic agonists, which stimulates sAPPa secretion (Allison *et al*., 2001; Lahiri *et al.,* 2002; Yogev-Falach, 2006). The dual mechanism of action of CHEIs and muscarinic agonists remains an attractive therapeutic intervention for Alzheimer's disease treatment. The dual action may be related to their direct or indirect interaction with 5'-UTR (5'-untranslated region) of APP mRNA and hence, 5'-UTR is considered as a novel drug target. Phenserine, a well known CHEI, has been shown to reduce the translation of APP by interacting with 5'-UTR, thereby reducing the levels of APP (Haass *et al*., 1999; Fisher, 2007).

BACE1 is an important and attractive drug target for the development of drug for the treatment of Alzheimer's disease. Since the discovery which was a little more than a decade ago, several research work supported the role of BACE1 in Alzheimer's disease (Chang *et al*., 2004; Vassar *et al.,* 2011). It is a single transmembrane protease involved in the amyloid cascade, which cleaves APP to form Aβ (Fig. 6). APP cleavage by BACE1 is the rate limiting step in the formation of Aβ, and hence it is an important

therapeutic target in the development of drug for the treatment of Alzheimer's disease (Chang *et al*., 2004; Chami *et al.,* 2012) and the same is discussed in the following section 2.7.2. GABAergic neurotransmission is considered as one of the potential target for the cognitive dysfunction in Alzheimer's disease (Nava-Mesa, 2014). Serotonin, their receptors and transporters play an important role in the aging and Alzheimer's disease (Mann and Yates, 1983; Rodriguez *et al*., 2012; Benhamu *et al*., 2014). Hence, blocking selective serotonergic system is found to be an effective symptomatic treatment in Alzheimer's disease and other related disorders, targeting cognitive deficiencies in specific (Rodriguez *et al*., 2012; Benhamu *et al*., 2014).

Tau phosphorylation and subsequent accumulation as NFTs is the hallmark of Alzheimer's disease. Therapeutic interventions which can inhibit tau phosphorylating kinases and enhances Protein Phosphatase (PP2A) activity are thought to be appropriate strategy in the treatment of Alzheimer's disease (Iqbal *et al*., 2005; Salomone *et al*., 2012; Ghezzi *et al*., 2013).

Research studies suggested that the treatment with Nonsteroidal anti-inflammatory (NSAIDs) drugs can reduce risk of Alzheimer's disease (Atack *et al*., 1986; Lahiri *et al.,* 2002). Celastrol, a potent anti-inflammatory compound could be a potential drug in the therapeutic intervention for Alzheimer's disease (Allison *et al*., 2001; Lahiri *et al.,* 2002). The recent studies have evoked a growing interest for an anti-inflammatory drug to treat Alzheimer's disease (Iwata *et al*., 2001).

Currently, research is more focused on new inhibitors for BACE1, PS-1 and γ-secretase. Additionally vaccination, antioxidants, anti-inflammatory agents and hormone therapy are also, being explored (Lahiri *et al.,* 2002)

2.7.2. Beta-secretase (BACE1) - A validated drug target for Alzheimer's disease:

BACE1 is an important and attractive target for the development of therapeutic intervention for Alzheimer's disease. Since the discovery, which was a little more than a decade ago, several research works supported the role of BACE1 in Alzheimer's disease (Chang *et al*., 2004; De Strooper, 2010). It is a single transmembrane protease involved

in the amyloid cascade, which cleaves APP to form Aβ (Zhang, 2012; Figure 6). In amyloid cascade, APP cleavage by BACE1 is a rate limiting step in the formation of Aβ, and hence, it is considered as an important therapeutic target in the development of drug for the treatment of Alzheimer's disease (Chang *et al*., 2004; Strooper, 2010; Chami *et al.,* 2012). BACE1 is a transmembrane aspartic protease, which is related to the family of pepsin and retroviral aspartic protease. BACE1 is a localized enzyme and found to be active in a low optimum pH (Vassar and Cole, 2007; Vassar *et al.,* 2009). The three dimensional catalytic domain of beta secretase was shown in Fig. 5. The development of BACE1 inhibitors was actively, pursued for the past several years and few are there in different stages of clinical development (Ghosh *et al.,* 2008). The BACE1 validation and characterization studies have demonstrated that BACE1 is endogenous secretase localized in the brain, which can become a promising therapeutic target (Luo *et al*., 2001; Strooper, 2010). Recent studies have shown that BACE1 knockout mice exhibit some of the abnormal phenotypes that are related to the physiological functions of BACE1. Due to this, there are several concerns about complete inhibition of BACE1 which may lead to severe adverse side effects (Cai *et al*., 2001). BACE1 is involved in processing of several other proteins along with APP and identifying these proteins is necessary for evaluating the possible toxicity, which arises due to the complete inhibition of BACE1. BACE1 gene deletion in mice led to only mild phenotypic changes, indicating that BACE1 can act as a therapeutic target (Strooper, 2010; Ghosh *et al.,* 2014). In another experiment, BACE1 knockout mice were found to be viable and fertile and showed no abnormal pathologies (Cai *et al*., 2001; Vassar and Cole, 2007; Ghosh *et al.,* 2014). These findings indicate that there are a least chances for severe side effects due to the inhibition of BACE1.

In a transgenic mice study, small interfering RNA attenuated both amyloidogenesis and cognitive deficits in transgenic mice (Cai *et al*., 2001) confirming the validation of BACE1 as therapeutic target for drug development for the treatment of Alzheimer's disease.

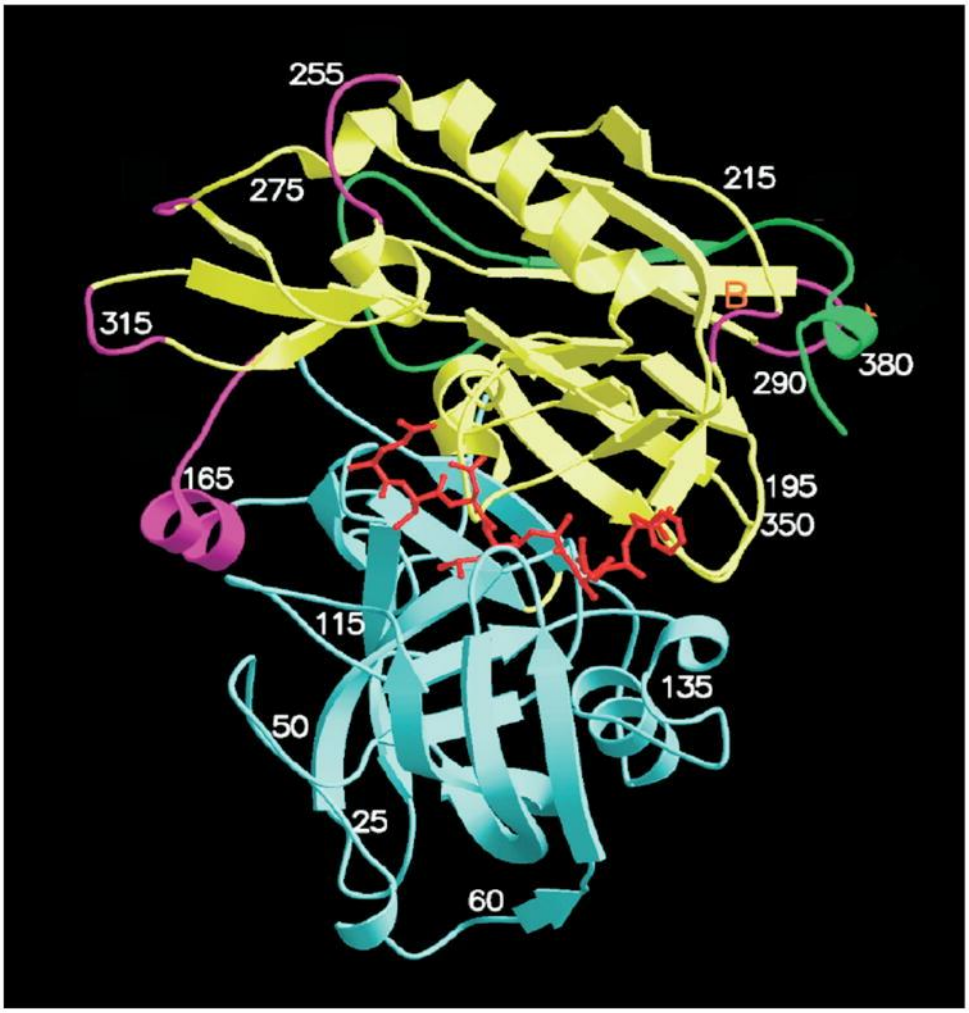

Figure 5. A ribbon model of BACE1 catalytic domain derived from its crystal structure (Source: Ghosh *et al.,* 2008)

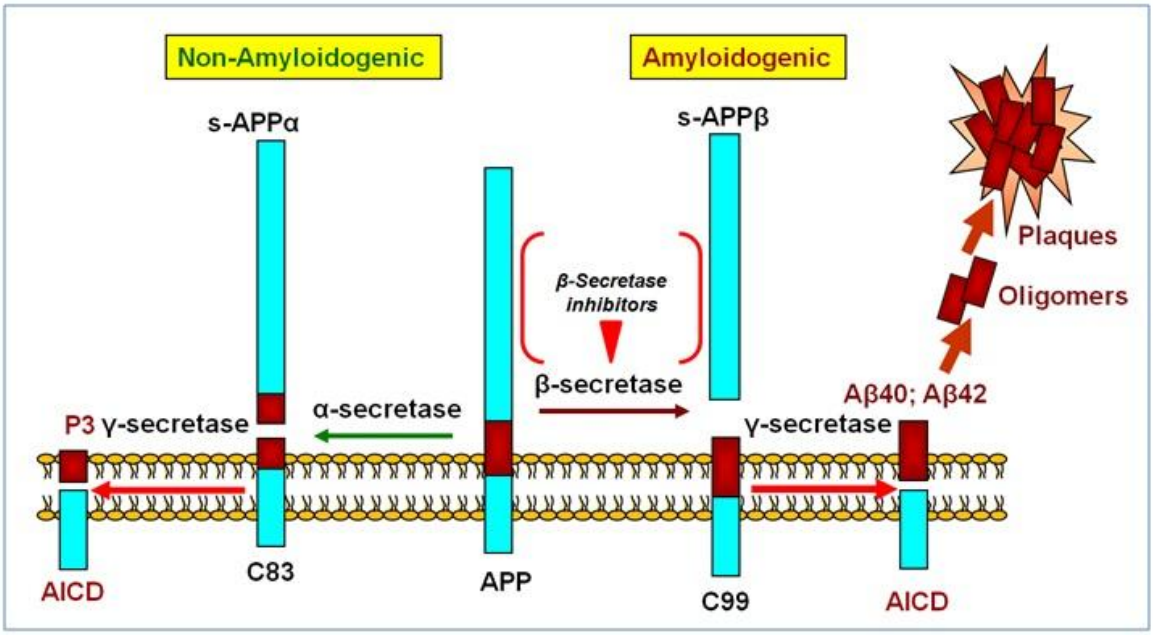

Figure 6. Picture showing the amyloidogenic and non-amyloidogenic cascade of APP processing. Inhibition of BACE1 can lead to the prevention of formation of Aβ proteins and their subsequent accumulation as Aβ plaques in Alzheimer's disease brain (Source; Zhang, 2012).

BACE1 is considered as a better therapeutic target than α and γ secretase (Vassar and Cole, 2007) for Alzheimer's disease. Targeting BACE1 for the development of drug for the treatment of Alzheimer's disease has several advantages viz, i) there is no alternative enzyme to initiate the amyloidogenic pathway in the absence of BACE1, thus inhibition of BACE1 leads to Aβ reduction and also eliminate the downstream steps of disease pathogenesis, ii) Based on the literature, there is no serious side effects due to the inhibition of BACE1 and iii) the chances of developing BACE1 inhibitor seems to be good. Several proof of concept studies confirmed that the BACE1 is one of the ideal target for the drug development (Ghosh *et al.,* 2008) for Alzheimer's disease.

Natural compounds, particularly those used as traditional medicines are ideal for the inhibition of BACE1 due to the higher safety profile (Zhang and Tanzi, 2012). Focusing on natural compounds for finding BACE1 inhibitor can lead to a potent drug candidate with no or minimal side effects. In this research work, BACE1 was selected as drug target against which natural compounds were screened and evaluated for the therapeutic application in Alzheimer's disease.

2.8 Current Treatment Options:

2.8.1 Modulating neurotransmission:

a. Acetylcholinesterase inhibitors:

The acetylcholinesterase inhibitors (ChEI) work by inhibiting the enzyme called acetylcholinesterase. It degrades the neurotransmitter called acetylcholine, which is involved in synaptic connectivity between neurons (Birks, 2006). The ChEI's are well accepted for the treatment of Alzheimer's disease, but they are symptomatic in nature. The ChEI's such as tacrine, galantamine, rivastigmine and donepezil (Nordberg *et al.,* 1998; Schneider, 2000; Patwardhan *et al*., 2009) are available for the treatment of Alzheimer's disease. Among these drugs, Tacrine, which was the first drug to be approved for the treatment of Alzheimer's disease, was removed from the US market due to hepatotoxicity (Lukiw, 2012). However, these drugs give relief from the symptoms associated with Alzheimer's disease but do not treat the cause. Research is on for the development of drug delivery systems, so that the drug molecules can efficiently pass through the Blood Brain Barrier (BBB) for better efficacy (Cedergren *et al*., 2007).

b. N-methyl D-aspartate (NMDA) receptor antagonists:

Glutamatergic neurotransmission regulate memory function in the brain through glutamate receptors such as alpha-amino 3-hydroxy methyl 4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartame (NMDA) receptors (Newcomer, 2000; Gasparini *et al.* 2013). Evidence suggests that the abnormal glutamatergic activity is associated with the pathophysiology of Alzheimer's disease. Inappropriate activation of N-methyl-D-aspartame receptors by glutamate leads to neuronal cell death and may cause cognitive decline associated with dementia (Newcomer, 2000; Wenk *et al.,* 2006; Weschules *et al*., 2008). Most AMPA receptors are impermeable to Ca^{2+} and contribute to fast synaptic transmission (Tukey, 2013). In contrast, NMDA receptors are characterized with high permeability to Ca^{2+} ions and slower gating kinetics (Wenk *et al.,* 2006; Malenka, 2010). NMDA receptors activation by released glutamate allows the influx of Na^{+} and Ca^{2+} ions, ultimately, leading to the postsynaptic excitation which affects learning skills (Wenk *et al.,* 2006). One of the NMDA antagonist 1-amino-3, 5-dimethyladamantane (memantine) is being used for the treatment of patients with

moderate to severe Alzheimer's disease (Wenk *et al.,* 2006) and is available under the brand name Namenda (Forest), Axura, Akatinol (Merz), Ebixa and Abixa (Olivares *et al.,* 2012). Memantine is also used for the treatment of Parkinson's disease, spasticity and also for cerebral disorders such as coma, cerebrovascular and age-related psychiatric issues (Wenk *et al.,* 2006). However, these groups of medicines do not treat Alzheimer's disease but slows down the disease progression.

c. *GABAergic* modulation:

GABAergic neurotransmission is considered as one of the potential target for the cognitive dysfunction in Alzheimer's disease (Nava-Mesa, 2014). In Alzheimer's disease brain, synaptic dysfunction caused by Aβ is linked to excitatory and inhibitory neurotransmission and the treatments based on modulation of these neurotransmission have shown to improve Alzheimer's disease symptoms. Gamma-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the central nervous system (CNS) which regulates the excitatory activity and is involved in the regulation of learning and memory (Nava-Mesa, 2014). Production of higher amount of GABA is related to the impairment of synaptic transmission. The enzyme glutamic acid decarboxylase synthesizes GABA from glutamate, which is later metabolized by the GABA transaminase (GABA-T). GABA is released into nerve terminals, which are subsequently metabolized into succinic acid and semialdehyde (Sasaki *et al.,* 1986; Lanctot *et al.,* 20014). Suppressing the release of GABA restores the synaptic plasticity, learning and memory functions in mice (Jo *et al.,* 2014). Several studies showed that the activity of glutamine synthetase is decreased in Alzheimer's disease specifically in astrocyte and may lead to cognitive impairment in Alzheimer's disease. Hence, targeting GABA metabolizing enzymes is considered as one of the therapeutic targets in developing drugs for neurological disorders such as Alzheimer's disease (Nava-Mesa, 2014). GABA synthesizing and metabolizing enzymes such as glutamic acid decarboxylase (GAD) and GABA-transaminase (GABA-T) are closely associated with Alzheimer's disease by decreasing the levels of GABA significantly in cortical areas of the brain (Lanctot *et al.,* 2014). In transgenic mice (hAPP), Levetiracetam, an antiepileptic drug was shown to reverse synaptic dysfunction, learning and memory and hence, can attenuate cognitive decline in Alzheimer's disease. Side effects due to long-

term use of these drugs limited their therapeutic application in human (Nava-Mesa, 2014).

d. Serotonin receptor modulation:

Serotonin (5-hydroxytryptamine) is one of the important neurotransmitter involved in many bodily functions such as regulation of intestinal movement, mood, appetite and sleep, in addition to cognitive functions such as memory and learning (Meltzer *et al.,* 1998; Rodriguez *et al*., 2012). Serotonin, their receptors and transporters play an important role in the aging and Alzheimer's disease (Mann and Yates, 1983; Rodriguez *et al*., 2012; Benhamu *et al*., 2014). Hence, blocking selective serotonergic system is found to be an effective symptomatic treatment in Alzheimer's disease (Rodriguez *et al*., 2012;Benhamu *et al*., 2014). However, these Selective serotonin reuptake inhibitors (SSRIs) did not show any benefits in Alzheimer's disease patients (Sepehry *et al.,* 2012).

2.8.2 Tau based therapies:

a. Tau Phosphorylation inhibition:

Tau is a microtubule bound protein involved in assembly and stability of microtubule in neuronal cells (Lim and Halpain, 2000). Hyperphosphorylation of tau leads to destabilization of microtubule and its aggregation as intraneuronal NFTs, which is one of the hallmarks of pathophysiology of Alzheimer's disease (Iqbal, 2004). Tau hyperphosphorylation reduces its affinity for microtubules leading to destabilization of neuronal cytoskeleton (Grundke-Iqbal, 1986). The solubility of the tau protein is affected by its hyperphosphorylation leading to its aggregation as paired helical structures (PHF) to ultimately form NFTs. Paired helical filaments (PHF) are important structural feature formed by the hyperphosphorylated tau proteins from the depolymerized microtubules (Luna-Munoz *et al.,* 2013; Figure 7). The aggregation of tau can lead to different types of dementias, in which both symptoms and onset of the disease depends on the impact of phosphorylation (Bennecib *et al*., 2000).

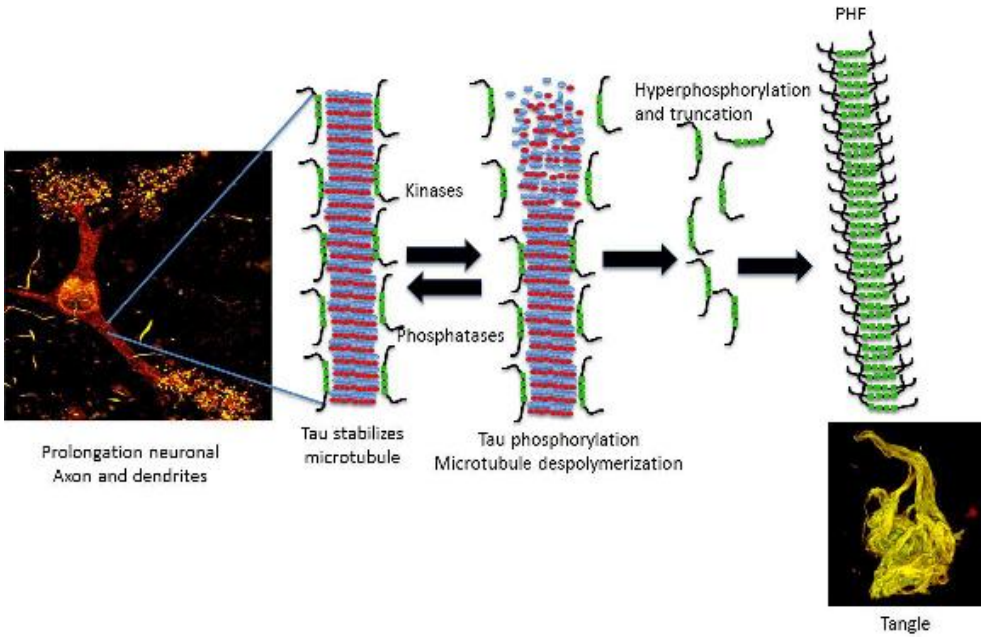

Figure 7. Tau based neurological changes during the onset of Alzheimer's disease (Source: Luna-Munoz *et al.,* 2013).

One of the main reasons for tau hyperphosphorylation seems to be the imbalanced activity of protein kinases. Protein Phosphatase-2 (PP2A) activity goes down during the pathophysiology of Alzheimer's disease leading to abnormal hyperphosphorylation of tau (Iqbal *et al*., 2005). Inhibitors of PP2A activity such as aluminium, were shown to induce hyperphosphorylation of tau, leading to the formation and accumulation of NFTs (Walton, 2012). Enhancing the activity of PP2A may alleviate the hyperphosphorylation of tau and prevent subsequent events of NFTs. Thus, therapeutic approaches that inhibit tau-phosphorylating kinases (glycogen synthase kinase-3 (GSK) and p70-S6-kinase) are thought to be one of the appropriate strategies in the treatment of Alzheimer's disease. In this regard, lithium, methylthioninium chloride (MTC) and valproate were used for the inhibition of protein kinases (Salomone *et al*., 2012; Ghezzi *et al*., 2013). Both lithium and valproate were found to inhibit GSK, whereas MTC was able to bind to the domain responsible for tau aggregation. MTC has a potential clinical use and awaits for phase III clinical trial. Treatment with Lithium demonstrated a significant decrease in concentrations of phosphorylated tau in CSF and better cognitive performance. Tideglusib, a GSK 3 inhibitor is under clinical evaluation (del Ser *et al.,* 2013). However, research is still in the infant stage to achieve the goal of application for the treatment of Alzheimer's disease using these strategies.

b. Microtubule stabilization:

Microtubules are key components of the cytoskeleton that are filamentous, long, tube-shaped protein polymers, which are essential for intracellular transport and cellular metabolism (Andreadis *et al*., 1992; Jordan and Wilson, 2004; Parker *et al.,* 2007). They are crucial in the maintenance and development of cell shape, cell signaling, intracellular transport and cell division (Goedert *et al*., 1988). A microtubule is heterodimeric in nature consisting of α and β-tubulins, which are arranged in the form of slender filamentous tubes, which can be many micrometers long. They are highly dynamic polymers α and β-tubulins and their dynamics in living cells are tightly regulated both temporally and spatially (Lindwall *et al*., 1984; Jain *et al,* 2013). Various regulatory proteins control the functional diversity of microtubules through several post-translational modifications of tubulin (Biernat *et al.,* 1993; Jordan and Wilson, 2004). The rationale behind microtubule-binding drug approaches in tauopathies is to stabilize the microtubules by reducing hyperphosphorylation of tau thereby preventing the formation of NFTs (Kowall *et al*., 1987; Caceres *et al*., 1990). Acetylated-tubulin is a marker for microtubule stability and levels were found to be reduced in Alzheimer's disease neurons. In a tissue culture experiment, hyperphosphorylation of tau resulted in transport impairment (Sydow *et al*., 2011). Hence, stabilization of microtubules is considered as one of the strategy to prevent Alzheimer's disease symptoms. Microtubule binding agents works by acting as substitute for loss of tau protein and restores the axonal transport (Ballatore *et al.,* 2012). Chemotherapeutic drug called Taxol and its analog found to stabilize microtubule (Schneider and Mandelkow, 2008; Kimura *et al*., 2010). In a Tg mice study, Taxol was found to increase the number of microtubules on neuronal cells (Bliss *et al*., 1993; Schneider and Mandelkow, 2008). In a triple transgenic mouse model, treatment with neuronal tubulin-binding agent, octapeptide NAP (Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln; NAPVSIPQ), reduced the levels of hyperphosphorylated tau, which in turn enhanced the cognitive function of these mice (Bliss *et al*., 1993; Grill *et al*., 2010).

c. Tau based immunotherapy:

An elevated level of tau oligomers was detected in the early stages of Alzheimer's disease. Like Aβ, tau aggregates are highly toxic to neuronal cells (Perrin *et al*., 2009). Tau oligomers induce mitochondrial dysfunction, synaptic loss, cell death and memory impairment. Hence, clearance of oligomeric tau is one of the strategies in therapeutic intervention for Alzheimer's disease. However, it could be difficult due to soluble tau aggregates located inside the neurons (Selkoe, 2001). Antibodies and vaccines are found to be effective in removing intracellular tau (Lemere *et al*., 2010). Immunization was found to be effective in reducing tau protein as well as delayed the onset of severe sensory motor deficits in the transgenic rats (Solomon *et al*., 1997; Wang *et al.,* 2012). Specific antibodies of tau when induced, were found to cross blood brain barrier and able to bind to tau proteins, which subsequently reduced tau aggregation and delayed progression of the Alzheimer's disease phenotype (Schenk *et al.,* 1999; Wang *et al.,* 2012). Immunization of transgenic mice with Tau379-408 (PSer396, 404) peptide prevented cognitive decline by reducing tau proteins in the brain (Gilman *et al.,* 2005). However, immunization with tau antibodies has significant potential risks. In conclusion, the mechanism about how tau immunotherapy work in an Alzheimer's disease patient remains elusive and studies need to be undertaken in this direction in order to better exploit tau immunotherapeutic strategy (Wang *et al.,* 2012).

2.8.3 Amyloid based strategies:

Aβ protein based strategies, which can reduce the level of Aβ peptide in the brain, are considered as very important in the therapeutic intervention of Alzheimer's disease (Ghosh, 2012). Drugs based on amyloid based strategies belong to the category of disease modulating drugs. The important amyloid based strategies are BACE1 inhibition, immunotherapy, prevention of Aβ aggregation and enhancing of Aβ clearance (Lemere, 2013). There are specific rationales behind usage of each of these strategies; at the same time, all of them have their own advantages, disadvantages and limitations. Hence, Aβ based therapies are still under investigation (Lansdall, 2014). Important obstacles with Aβ based therapies include non-specificity, worsening cognitive decline and safety issues (WHO, 2013). Attempts are being made to these strategies to make them more efficient for therapeutic application by removing

unwanted auxiliary effects. Nevertheless, the amyloid based therapies have high potential in the treatment of Alzheimer's disease. Hence, a detailed review of literature was done to understand these strategies, the mechanisms of these approaches and possibility of selecting one of the targets from these known strategies for the present research work.

a. Inhibition of BACE1:

BACE1, an aspartic protease, is one of the important targets in the therapeutic intervention for Alzheimer's disease. It is involved in one of the rate limiting step in enzymatic cleavage of APP in amyloidogenic pathways. APP is processed in both amyloidogenic (AG) and non-amyloidogenic (NAG) pathways, where α–secretase involved in initiating non-amyloidogenic pathway and amyloidogenic pathway is initiated by BACE1 (Zhang, 2012; Fig. 5). Amyloidogenic pathway leads to the production of toxic Aβ which subsequently aggregates to form senile plaques, an important manifestation of the Alzheimer's disease onset (Ghosh *et al.,* 2008) affecting cognitive functions. Inhibition of BACE1 may reduce the Aβ production thereby reducing the Alzheimer's disease pathogenesis (Menting and Claassen, 2014). Among the amyloid based therapies, inhibitors of BACE1 occupy an important position as disease modulating drugs. Before discussing the drugs and their mechanisms of action, it is necessary to understand APP processing pathways that lead to Aβ production. Since, significant details were given in the previous sections on neuropathologic conditions and drug targets based on BACE1, a brief of the basics will be discussed and then the related drugs will be described.

The thiazolidinedione compounds such as pioglitazone and rosiglitazone are found to inhibit BACE1 by stimulating the nuclear peroxisome proliferator-activated receptor γ (PPARγ) (Matsumura *et al.,* 2012; Reitz, 2012). Activation of PPARγ receptors, in turn, can negatively control the expression of BACE1 and APP. Apart from the direct effect on BACE1, therapeutic effects of these compounds in Alzheimer's disease could be achieved by their effect on insulin action (Reitz, 2012). Pioglitazone can cross the BBB, but it was not sure about the ability of rosiglitazone to reach internal brain regions. However, these BACE1 inhibitors showed considerable side effects in treated individuals (Reitz, 2012; Salomone *et al*., 2012). BACE1 inhibitor called CTS-21166

was found to be well tolerated and reduced plasma Aβ concentrations in mice and in phase I clinical trial (Ghosh, 2012).

Peptidomimetic drugs were found to be very good BACE1 inhibitor in *in vitro* studies but they suffer from poor pharmacokinetic properties. Hence, the research focus turned from large molecules towards small molecules which can efficiently cross BBB (Vassar, 2014). The major drug developmental hurdles such as selectivity and BBB penetration were overcome by many small-molecules and nonpeptide BACE1 inhibitors (Ghosh & Tang, 2015). Also, many such inhibitors have shown simultaneous reduction of brain Aβ and prevention of cognitive decline in animal model studies, and several BACE1 inhibitors are now in clinical trials. List of small molecules which are currently, in different stages of development are given in Table.1

Table 1. List of BACE1 inhibitors currently in the different stages of clinical trial (Menting and Claassen, 2014; Vassar, 2014)

Sl No	Drug Name	Name of the innovator company	Clinical Phase	Current status
1	AZD3293	AstraZeneca/Lilly	Phase 2/3	Ongoing
2	CTS-21166	CoMentis	Phase 1	Phase 1 completed
3	E2609	Eisai/Biogen Idec	Phase 2	Not Available
4	HPP854	High Point	Phase 1	Not available
5	MK-8931	Merck	Phase 2/3	Ongoing
6	PF-05297909	Pfizer	Phase 1	Not available
7	TAK-070	Takeda	Phase 1	Not available
8	LY2886721	Eli Lilly & company	Phase 2	Discontinued due to hepatotoxicity
9	VTP-37948	Vitae/Boehringer Ingelheim	Phase 1	Molecule kept on hold due to observed side effects (Skin rashes) during clinical trial

BACE1 inhibitors are known to decrease the production of Aβ from APP by inhibiting BACE1, and may not affect the Aβ plaques which are already deposited in the brain. Aβ deposition were seen at least a year before the occurrence of cognitive deficits or any phenotypic changes. This implies that BACE1 inhibitors can be used for preventive rather than curative strategy in the treatment of Alzheimer's disease. Now, a major challenge is to decide when to administer the BACE1 inhibitors to Alzheimer's disease patients (Vassar, 2014). Besides, overall understanding on BACE1 inhibitors is in early stage and lot of research aspects remains to be elusive. However, future research may ensure and reveal these inhibitors with higher efficiency and safety. Hence, BACE1 was

selected as a drug target in the present study against which natural plant based compounds were screened and evaluated for the therapeutic application in Alzheimer's disease.

b. Promoting amyloid clearance:

Aβ is generated by sequential enzymatic cleavages of APP by BACE1 and subsequently γ-secretase (McHugh *et al.,* 2012). Aβ clearance from the brain either by transportation or by degradation was found to be an important aspect in accumulation and formation of senile plaques. Hence, enhancing Aβ clearance is considered as important strategy for developing therapeutic interventions for Alzheimer's disease. In normal brain, Aβ synthesis and clearance was found to be 7.6% and 8.3% as measured in cerebrospinal fluid. Normally, Aβ clearance takes place through passive diffusion, interstitial fluid (ISF), uptake by microglial or astrocytic phagocytosis, clearance receptors and localized p-glycoprotein (Yoon and AhnJo, 2012). The structure of Aβ peptide shows that, it cannot be cleared rapidly from CNS crossing BBB. Only 10% of total Aβ can be cleared by passive diffusion and nonspecific clearance (Zlokovic, 2000; Selkoe, 2001; Miners *et al*., 2008). The endogenous receptor, Low-density lipoprotein receptor-related protein (LRP1) plays a major role in Aβ sequestering and transport across BBB from brain to blood (Deane *et al.,* 2009). In Alzheimer's disease brains, expression of low-density lipoprotein receptor-related protein (LRP1), which mediates Aβ transportation from the brain to blood, is down-regulated (Boulanger *et al*., 2007; Yoon and AhnJo, 2012). Vaccination or immunization is found to reduce the Aβ burden in Tg mice by sequestration of Aβ by an anti-Aβ antibody (Hallier Vanuxeem *et al*., 2009). Nonspecific flow of interstitial fluid (ISF) can carry Aβ passively into the CSF across the permeable ependyma of brain ventricles and from cerebral spinal fluid back to blood through the arachnoid granulations (Yamada *et al*., 2008).

c. Preventing amyloid aggregation:

Several strategies are being explored to prevent the formation of Aβ plaques, such as preventing the formation of Aβ by inhibition of BACE1 and its clearance (Chiti *et al*., 2006). There are several small molecules developed and tested for the application in preventing the Aβ aggregation, however, none of them reached the market (Ritchie *et al*., 2003; Neddenriep *et al.,* 2011).

2.8.4 Oxidative stress reduction:

Oxidative stress due to overproduction of free radicals is responsible for aging and several human diseases, including Alzheimer's disease (Zhao and Zhao, 2013). Metabolism of excitatory amino acids, metals and neurotransmitters in the brain produces high amount of reactive oxygen species (ROS), causing oxidative stress. ROS damages glial cells and neurons leading to neuronal degeneration or cell death (Padurariu *et al.,* 2013). Mitochondrial respiratory chain reactions and Aβ are found to be main source of accumulation of ROS in the Alzheimer's diseased brain, which is evident by the presence of higher levels of lipid peroxidation (Reed, 2011). Mitochondria are vulnerable to oxidative stress leading to the mitochondrial dysfunction, which is one of the important factors in the pathophysiology of Alzheimer's disease (Padurariu *et al.,* 2013; Zhao and Zhao, 2013). Oxidative stress is also linked to tau pathology in Alzheimer's disease (Zhao and Zhao, 2013). Malondialdehyde (MDA), a toxic byproduct of peroxidation causes protein oxidation (Padurariu *et al.,* 2013) in the brain which adds to the oxidative stress. There are several bodily antioxidant mechanisms such as antioxidant enzymes namely; superoxide dismutase, catalase, and glutathione reductase and glutathione peroxidase which effectively remove free radicals. Non-enzymatic antioxidants such as ascorbic, lipoic acid, polyphenols, carotenoids and chelating agents effectively quench produced ROS in the brain and provide protection from oxidative stress. However, brain has less antioxidant activity compared to other tissues in the body, making it as highly vulnerable part of the body (Uttara, 2009). In human clinical trials, antioxidants were failed in treatment cognitive disorders (Padurariu *et al.,* 2013) which may be due to their inability to cross blood brain barrier (Uttara, 2009).

2.8.5 Anti-inflammatory therapy:

Alzheimer's disease pathology is strongly associated with inflammatory reactions (Joshi *et al,* 2015) induced by various factors such as Aβ plaques and oxidative stress. Chronic inflammation in the nerve cells is one of the important pathophysiological changes seen in Alzheimer's disease. Major inflammatory changes in the Alzheimer's disease brain were found to be microgliosis, astrocytosis and presence of pro-inflammatory cytokines which accompany the Aβ plagues in Alzheimer's disease (Mishra *et al.,* 2008). Higher

level of tissue pro-inflammatory cytokines such as interleukins-1 β (IL-1β), tumor necrosis factor α (TNFα), and interferon γ (IFNγ) were found to increase the Aβ peptide and tau phosphorylation (Dyall, 2010; Joshi *et al*., 2015). Interleukins-1 (IL-1) induces the oxidative stress causing lipid peroxidation, activates microglial cells to produce inflammatory cytokines and increases the synthesis of APP (Dyall, 2010). Anti-inflammatory drugs such as diclofenac, fenprofen, meclofenamate and flurbiprofen were found to be helpful in Alzheimer's disease. These drugs inhibit COX-2, which is one of the major proinflammatory enzyme involved in inflammatory responses (Aggarwal *et al*., 2006; Anekonda, 2006). Prolonged use of anti-inflammatory drugs was found to be beneficial (Mishra *et al.,* 2008) in Alzheimer's disease. New generations of COX-2 specific inhibitors, which appears to be effective to control arthritis, are also considered as treatment options for Alzheimer's disease (Aisen *et al*., 2002).

Another proinflammatory enzyme, 5-lipoxygenase (5-LOX), which produces pro-inflammatory Leukotrienes from arachidonic acid is known to be associated with Alzheimer's disease. In an *in vitro* study, 5-LOX knockout cells were shown to have reduced production of Aβ and the similar effect was seen in transgenic mice with 5-LOX knockout. 5-LOX inhibition is also associated with improvement in learning and memory (Joshi *et al.,* 2015). Selective inhibitors of 5-LOX such as Zileuton was found to reduce the Aβ deposition significantly in the brains of Transgenic mice (Tg2576). This indicates that selective inhibition of 5-LOX is a novel therapeutic approach in the therapeutic intervention for Alzheimer's disease (Chu *et al.,* 2011).

During recent years, anti-inflammatory drugs were found to reduce Aβ aggregation (Aisen *et al*., 2002). The effect of NSAIDs in decreasing beta amyloid secretion was observed in cell cultures. However, mechanism of action for NSAIDs in reducing the levels of Aβ is not yet understood (Kukar and Golde, 2008). Besides, the results from various controlled clinical trials do not show any beneficial effect of NSAID's. NSAID's are known to cause side effects such as gastrointestinal ulceration (Dhikav *et al.,* 2003) limiting their long-term usage in the progressive neurodegeneration diseases such as Alzheimer's disease.

2.9 Problems associated with current treatment system:

Alzheimer's disease progression causes neuronal cells death leading to cognitive disability. Though there is no cure for Alzheimer's disease, there are five prescription drugs, which can slow the progress of Alzheimer's disease (Brookmeyer *et al*., 2007). Symptomatic treatments help in slowing down the cognitive decline for a limited period of time (Selkoe, 2001). These drugs have the disadvantage of being symptomatic, ignoring the fundamental mechanisms involved in the disease onset. In this view, disease modifying drugs will have a significant role in the future. One category among the disease modifying drugs is BACE1 inhibitors, hence this BACE1 was utilized as a drug target in the present thesis study. Most of the synthetic drugs are known to have side effects associated with them. As a solution to this issue, set of natural plant-based compounds were selected for this study through an extensive literature survey, which are subsequently used in *in silico*, *in vitr*o and *in vivo* studies in the present study. The final and important concern in the CNS drugs is the difficulty in delivering into the brain by crossing BBB, which could be resolved by suitable formulation development. In conclusion, the present thesis work would attempt to substantiate the need of a natural compound for the treatment of Alzheimer's disease starting from screening of compounds through literature survey till *in vivo* study.

2.10 Natural compounds in the treatment of Alzheimer's disease:

Natural compounds or phytochemicals are found to be the safe alternative to existing synthetic drugs due to minimal or low toxicity in humans. There are no drugs available for the complete cure of Alzheimer's disease. Currently, the approved drugs are symptomatic in nature and many of the synthetic drugs are not making their way into the market mainly due to toxicity associated with them. In the current situation, natural compounds are best alternatives to the synthetic drugs. Nearly 80% of the current drugs either have their origin from natural source or derived from natural source (Sundaram *et al.,* 2014). Several phytochemicals were identified and explored extensively as a rich source of lead compounds in the treatment of human disease such as anticancer,

antimicrobials and anti-inflammatory drugs. In ancient days, the whole plant extract was used for the treatment and the technological advancement led to the standardized extracts or purified phytochemicals (Sripathy *et al.,* 2015) with enhanced efficacy. Many of such compounds from natural resources are known to have neuroprotective and/or memory enhancing activity, which can be further explored for therapeutic application in brain diseases. Recently, discovered drugs from natural source for the treatment of neurodegenerative disorders like Alzheimer's disease and Parkinson's disease (PD) are listed in Table 2.

Table 2. Natural compounds and their derivatives in clinical development

S. No.	Name	Biological origin	Function	Reference
1	7 beta-Hydroxyepiandrosterone (HF-0220)	From adrenal hormone, Dehydroepiandrosterone (DHEA)	Neuroprotection	Dudas *et al.,* 2004 Sundaram *et al.,* 2014
2	Bryostatin-1	*Bryozoans Bugula neritina L*	Activates α-secretase and reduced Aβ level in Tg mice	Brahmachari, 2011 Sundaram *et al.,* 2014
3	Curcumin	*Curcuma longa* L.	Inhibition of Aβ protein aggregation, anti-inflammatory activity, antioxidant activity and inhibition of activities of the BACE1 and acetyl cholinesterase	Hamaguchi and Yamada, 2010 Sundaram *et al.,* 2014
4	Cyclosporin A (CsA)	*Tolypocladium inflatum*	Neuroprotective	Alessandri *et al*., 2002 Sundaram *et al.,* 2014
5	Huperzine A	*Huperzia serrata*	Inhibition of enzyme acetylcholinesterase	Brahmachari, 2011 Sundaram *et al.,* 2014 Ha *et al.,* 2011
6	Resveratrol	*Blueberries*	Resveratrol reduces the amount of Aβ peptides and promotes	Sundaram *et al.,* 2014

			intracellular degradation of Aβ.	Marambaud *et al*., 2005 Brahmachari, 2011 Pallas *et al,* 2009
7	Rifampicin	*Amycolatopsis rifamycinica Bala*	Anti-inflammatory and anti-aggregation of Aβ protein	Brahmachari, 2011 Sundaram *et al.,* 2014
8	ZT-1	*Huperzia serrata* (Huperzine A derivative)	Inhibition of enzyme acetylcholinesterase.	Ishiuchi *et al.,* 2013 Sundaram *et al.,* 2014

2.11 Summary:

This chapter is an extensive literature review of earlier research on Alzheimer's disease. This chapter briefs on Alzheimer's disease, its prevalence, pathophysiology, treatment options available, problems associated with the current treatments and the importance of phytochemicals in the treatment of neurological disease.

The information in this chapter shows that, the economic burden due to Alzheimer's disease is very high and currently, there are no treatments available for complete cure of disease. This necessitates the need for alternative therapeutic intervention to reduce the socio-economic burden. Earlier research supports phytochemicals as an alternative therapeutic intervention to synthetic drugs due to toxicity associated with latter. In the present research work, phytochemicals with neuroprotective and/or memory enhancing activity were short-listed and evaluated in *in silico, in vitro* and *in vivo* experiments, to short-list the potential therapeutic compound for the treatment of Alzheimer's disease.

Chapter-3

3. Short-listing natural compounds for *in silico* screening

3.1 Introduction:

Natural compounds such as phytochemicals are well proven to have diverse and effective bioactivity. Phytochemicals from several plants were identified and explored extensively as rich source of lead compounds in the treatment of human disease such as anticancer, antimicrobial and anti-inflammatory drugs (Tariq and Reyaz, 2013). In ancient days, the whole plants were used for the treatment and the technological advancement lead to the use extracts of specific plant parts. Subsequently, this lead to evolution of medical systems called ayurveda, siddha and unani in India, which is being followed even today (Sripathy *et al.,* 2015). Many of such compounds from natural resources are known to have neuroprotective and/or memory enhancing activity, which can be explored further for therapeutic application in brain diseases. One of the major limiting factors for development of these compounds into drug molecule is that, most of their site of action and mechanism of action is still unknown. Hence, one of the objectives of this study is to identify such plants/compounds with neuroprotective and memory enhancing property, and elucidate its site of activity using *in silico* tools. As a first part of the study, a thorough literature review of traditional knowledge and scientific research report was carried out and neuroactive-compounds from plants and marine sources were short-listed for *in silico* and *in vitro* studies.

3.2 Methodology Adopted:

The literature search mainly focused on traditional knowledge and scientific reports available on herbal plants. Literature collection was undertaken through a systematic search on the World Wide Web through the common search engine ‘Google’ and it’s ‘Google Scholar’. Literatures were also obtained from Science direct, Springer, Nature

publication and other open access journals. The major key words used for the search are phytochemicals and marine resources, Alzheimer's disease, neurological disorder, brain health, neuroprotective activity and memory enhancement.

Collected literatures were reviewed for compounds from plants and marine resources with neuroprotective and memory enhancing bioactivity. Compounds which have strong anti-oxidant, anti-inflammatory activity and those with potential to prevent nerve cell damage can be considered as neuroprotective compounds. Compounds with above mentioned bioactivities were short-listed. Then, in-depth information on each selected compounds, both from herbal and marine resource, were collected. Short-listed molecules were further taken for *in silico* screening studies.

3.3 Results:

Literature review results are consolidated in Tables 2A and 2B. List of compounds and its neuro-therapeutic role reported in literature and reference information's are consolidated in the Table 3, whereas details of natural compound given in Table 4.

Table 3. List of natural compounds short-listed for *in silico* screening

Sl. No	Natural molecule	Description	References
1	6-hydroxyflavone	6-Hydroxyflavone (6-HF) is a naturally occurring flavonoid. It is found leaves of common Acanthaceae *Barleria prionitis* Linn. It is a selective inhibitor of $GABA_A$ receptor subtype, which has a therapeutic role in Alzheimer's disease.	Ren *et al.,* 2010
2	Acetyl-L-Carnitine	Acetyl-L-carnitine is naturally present in the human body. It is involved in modulation of brain energy, synaptic morphology and synaptic transmission. The Phase II clinical trial was done with Acetyl-L-Carnitine containing nutraceutical formulation and was found to improve cognitive performance.	Pettegrew *et al.,* 2000 Remington *et al.,* 2015
3	Allicin	Allicin is an organosulfur compound from Garlic. Administration of allicin prevents learning and memory impairment in Alzheimer's disease model mice, by increasing the activity of SOD and decreasing levels of	Li *et al.,* 2010

		malondialdehyde as well as expression of Aβ and p38 MAPK in the brain.	
4	Asthaxanthin	Asthaxanthin is a carotinoid pigment occurs naturally in plant and animal. Asthaxanthin is a strong antioxidant and was shown to enhance the memory.	Katagiri *et al.,* 2012
5	Bis-demethyl curcumin (BMC)	BMC is a natural metabolite of Curcumin. It was shown to have potent anti-inflammatory and anti-oxidant activity.	Ravindran *et al.,* 2010 Loizzo *et al.,* 2012 Liu *et al.,* 2010
6	Capsaicin	Capsaicin significantly attenuated cold water stress (CWS)-induced spatial memory impairment and prevented tau hyperphosphorylation by preventing the inhibition of protein phosphatase 2A (PP2A).	Jiang *et al.,* 2013
7	Catechin	Catechin is a naturally occurring phenol, iron chelator and antioxidant, which is found in many plants. Catechin was found to prevent Aβ-induced neuronal cell death in PC12 cells.	Heo and Lee, 2005
8	Chlorogenic acid	Chlorogenic acid (CGA), a phytochemical, is an ester of caffeic acid and (−)-quinic acid, mainly found in coffee. In an *in vitro* and *ex vivo* experiment, Chlorogenic acid was found to inhibit acetylcholinesterase activity, which is one of the risk factor in Alzheimer's disease.	Kwon *et al.,* 2012 Eskelinena, and Kivipelto, 2010
9	Cinnamic acid	Cinnamic acid, which is present in oil of cinnamon, is a potential antioxidant. It is known to attenuate protein oxidation and also protein aggregation, which might be having therapeutic application in Alzheimer's disease.	McIntyre *et al.,* 2015 Adisakwattana *et al.,* 2012
10	Coumaric acid	Coumaric acid is present in many fruits and vegetables. It was found to be effective against Aβ25−35-induced toxicity in PC12 cells by significantly inhibiting the expression of iNOS and COX-2.	Yoon *et al*., 2014
11	Creatine	Creatine is an amino acid found naturally in many animal sources. It is involved in energy metabolism in the brain tissue and was considered as a potential therapeutic intervention for Alzheimer's disease	Gallant et al., 2006
12	Curcumin	Curcumin, a principle component of	Ono *et al*., 2004

		Indian spice turmeric (*Curcuma longa*), showed anti-amyloidogenic activities by dose-dependent inhibition of Aβ plaque formation.	Ringman *et al.,* 2005
13	Ellagic acid	Ellagic acid, a commonly found polyphenol of fruits and nuts, significantly reduced Aβ-induced neurotoxicity in SH-SY5Y cells. Aβ is one of the hallmarks of pathogenesis of Alzheimers disease.	Feng *et al.,* 2009
14	Emodin	Emodin is a phytochemical present in traditional Chinese medicinal herb, *Polygonum cuspidatum.* It is shown to have neuroprotective activity in cultured cortical neurons by attenuating Aβ-induced neurotoxicity.	Liu *et al.,* 2010
15	Ferulic Acid	It is a phenolic compound found in the plant cell wall. The chemical structure resembles Curcumin. It is a potent antioxidant, anti-inflammatory agent and was found to suppress Aβ induced neurotoxicity.	Yan *et al*., 2001
16	Fisetin	Fisetin, a common component of fruits such as strawberries was shown to inhibit Aβ fibril formation in *in vitro* assay.	Akaishi *et al.,* 2008
17	Genistein	Genistein is a most active component of soy isoflavone. It has strong antioxidant activity, hence may be useful in preventing oxidative damage of brain cells.	Wei *et al*., 1996
18	Hesperetin	Hesperetin, a natural flavanone from citrus fruits, is a potent anti-oxidant. It was shown to protect cultured cells by attenuating $A\beta_{25-35}$-induced neuronal damage.	Cho, 2006
19	Hippeastrine	Hippeastrine is an alkaloid present in maryllidaceae family. Considering that it is similar to the alkaloid, galanthamine which has been used to Alzheimer's disease treatment, Hippeastrine is considered for *in silico* evaluation.	Pagliosa *et al.,* 2010
20	Hordenine	Hordenine is a tyramine-type alkaloid found in many plants. This component is found in many traditional plants used for Alzheimer's disease treatment e.g. *Leucojum aestivum.*	Georgieva *et al.,* 2007
21	Hypericin	Hypericin, a naturally found plant based	Taniguchi *et al.,*

		polyphenol, inhibits both tau protein and Aβ in *in vitro* experiment.	2005
22	Hyperoside	Hyperoside is a bioactive flavonoid compound isolated from Hypericum perforatum. Hyperoside was shown to protect Aβ-induced cortical neurons via mitochondrial apoptotic pathway and proposed as one of the potential compound for the treatment of Alzheimer's disease. It acts through restoring the mitochondrial function.	Zeng *et al.,* 2011 Li *et al*.,2008
23	Indole-3-carbinol	Indole-3-carbinol is a glucosinolate found in cruciferous vegetables. This can cross blood brain barrier (Abcam product data sheet, Indole-3-carbinol (I3C) ab143132) and may have neuroprotective activity.	https://en.wikipedia.org/wiki/Indole-3-carbinol
24	Isoquercitrin	Isoquercitrin is a flavonoid commonly found in plants. It is shown to have neuroprotective activity in Alzheimer's disease.	Magalingam *et al.,* 2015
25	Kaempferol	Kaempferol reverse Aβ-induced impaired performance of PC12 cells in Y-maze test.	Kim *et al.,* 2010
26	Levodopa	Levodopa is an amino acid present naturally in many plant and animals and the main source is Mucuna bean. Natural extracts containing Levodopa are being sold as dietary supplement for the prevention and treatment of neurological disease.	
27	Limonene	Limonene, is a natural cyclic monoterpene extracted from citrus peels (Pubchem Compound Summary for CID 440917). It was shown to have anti-stress properties and hence short-listed for the study	https://pubchem.ncbi.nlm.nih.gov. http://confidenceanalytics.com/terpenes
28	Lutein	Lutein is a carotenoid. It was shown to be helpful in lowering risk of Alzheimer's disease mortality in adults.	Kiko *et al*., 2012
29	Luteolin	Luteolin is a phytochemicals present in *Perilla frutescens* var. *acuta.* It was known to *inhibit* BACE1, an enzyme involved in pathophysiology of Alzheimer's disease.	Choi *et al*., 2008
30	Lycopene	Lycopene is a carotenoid present in red vegetables such as tomatoes and red	Qu *et al.,* 2011

		fruits. It was shown to attenuate Aβ-induced neurotoxicity in cultured rat cortical neuronal cells.	
31	Lycorine	Lycorine is an alkaloid found in the medicinal plants along with other important alkaloids. Lycorine is a weak acetylcholine esterase inhibitor. Slight modification of this alkaloid improves the activity many folds.	Pinho *et al.,* 2013
32	Mollugin	Mollugin is a phytochemical isolated from *Rubia Cordifolia L*. It is known for its neuroprotective effects	
33	Morin	Morin, one of the natural polyphenols, can dose-dependently destabilize the preformed Aβ fibrils as shown in the *in vitro* assay.	Ono *et al.,* 2003
34	Myricetin	Myricetin is a flavanoid which modulates NMDA receptor resulting in reduced glutamate induced Ca(2+) overload. It was also shown to inhibit ROS production caused by glutamate. In addition, it up-regulates the activity of α-secretase and directly binds to BACE1and inhibits it resulting in significant decrease in Aβ formation.	DeToma *et al*., 2011
35	Naringenin	Naringenin (40,5,7-trihydroxyflavanone), a flavonoid present in grapefruit juice, could ameliorate Alzheimer's disease-type neurodegeneration with cognitive impairment (AD-TNDCI) in a rat model.	Badruzzaman, *et al.,* 2012
36	Physostigmine	Physostigmine is an alkaloid from the calabar bean (*Physostigma venenosum).* It was shown to restore acetylcholine efflux from Alzheimer's brain slices and also a well-known Acetylcholine esterase inhibitor.	Christie *et al*., 1981 Nilsson *et al.,* 1986
37	Phytic acid	Phytic acid was shown to have neuroprotective effects in 6-hydroxydopamine- (6-OHDA-) induced Parkinson's disease in a cell culture model. The efficacy of phytic acid in Tg2576 mouse model of Alzheimer's disease was studied and shown to be beneficial.	Xu *et al*., 2011 Ashton Acton, 2012
38	Pregnenolone	Pregnenolone is an endogenous steroid hormone, found to be a safe drug for the treatment of Alzheimer's disease. It has	Roberts, 1995

		strong anti-inflammatory activity.	
39	Puerarin	Puerarin is a flavonoid isolated from plant Pueraria. It is known for its therapeutic application Alzheimer's disease.	Zhou *et al.,* 2014
40	Resveratrol	Resveratrol reduces the amount of secreted and intracellular Aβ peptides. Resveratrol also promotes intracellular degradation of Aβ.	Marambaud *et al*., 2005
41	Rosmarinic acid	Rosmarinic acid a natural compound present in *Perilla frutescens* var. *acuta* extract. It was shown to inhibit BACE1, an enzyme involved in Alzheimer's disease pathophysiology.	Choi *et al*., 2008
42	Silibinin	Silibinin is an active ingredient of milk thistle (Silybum marianum). It was shown to inhibit Aβ-protein fibril formation and neurotoxicity in PC12 cells. In the *in vivo* study involving APP Tg mice, it was shown to reduce A β deposition in the brain and also improvement in behavioral abnormalities.	Murata *et al.,* 2010
43	Tazettine	Tazettine is an alkaloid extracted from the bulbs of *Galanthus* Species. It is also present in the bulbs of *Narcissus tazetta* and many other plant species such as Amaryllidaceae. It was shown to have anticholinesterase activity.	Sourmaghi *et al.,* 2010 Takos *et al.,* 2013
44	Tetramethylpyrazine	Tetramethylpyrazine (TMP) is an alkaloid present in the Ligusticum chuanxiong hort root extract. It was shown to reduce oxidative stress and attenuate cell death in neuronal cultures. TMP is also shown potent antioxidant, anti-inflammatory, and anticancer properties. TMP significantly inhibited the inflammatory response stimulated by Aβ peptide in microglial cells. It also suppressed the NF-κB activation induced by Aβ indicating its therapeutic role in the treatment of Alzheimer's disease.	Weixia Li *et al.,* 2012 Kim *et al.*, 2014
45	Vinpocetine	Vinpocetine is an alkaloid obtained from the periwinkle plant *Vinca minor*. It was shown to possess neuroprotective and antioxidant properties. It was also shown to increase the blood flow to the	Medina, 2010 Patyar *et al*., 2011

		brain.	
46	Wogonin	Wogonin (5,7-dihydroxy-8-methoxyflavone) is a phytochemical isolated from *Scutellaria baicalensis*. Wogonin exhibits anti-inflammatory activity thereby effective in preventing Alzheimer's disease.	Park, 2010 He *et al.,* 2012
47	Xanthorrhizol	Xanthorrhizol is an antioxidant and potential neuroprotective agent. It was found to attenuate glutamate-induced neurotoxicity in the murine hippocampal HT22 cell line.	Lim *et al*., 2005

Table 4. Details of natural compounds short-listed for *in silico* screening

Sl. No	**Name of compound**	**Type of compound**	**Molecular formula**	**Molecular Mass g/mol**	***Source***
1	6-hydroxyflavone	Flavonoid	$C_{15}H_{10}O_3$	238.24	*Barleria prionitis Linn*
2	Acetyl-L-Carnitine	Amino acid (derivative of Carnitine)	$C_9H_{17}NO_4$	203.236	Beans
3	Allicin	Organosulfur compound	$C_6H_{10}OS_2$	162.26	Garlic
4	Astaxanthin	Terpene	$C_{40}H_{52}O_4$	596.841	Microalgae
5	Bis-demethyl curcumin (BMC)	Phenolic compound		340	*Curcuma Longa*
6	Capsaicin	Vanilloids	$C_{18}H_{27}NO_3$	305.41	Chili pepper
7	Catechin	Flavonoid	$C_{15}H_{14}O_6$	290.26	Green Tea
8	Chlorogenic acid	Phenolic compound	$C_{16}H_{18}O_9$	354.31	Coffee
9	Cinnamic acid	Unsaturated carboxylic acid	$C_9H_8O_2$	148.1586	Number of plants
10	Creatine	Nitrogenous organic acid	$C_4H_9N_3O_2$	131.14	Meat
11	Curcumin	Phenolic compound	$C_{21}H_{20}O_6$	368.39	*Curcuma Longa*
12	Ellagic acid	Phenolic compound	$C_{14}H_6O_8$	302.197	Berries
13	Emodin	Anthraquinone	$C_{15}H_{10}O_5$	270.24	*Aloe vera*
14	Ferulic Acid	Phenolic compound	$C_{10}H_{10}O_4$	194.18	Plant cell wall
15	Fisetin	Flavonoid	$C_{15}H_{10}O_6$	286.2363	Many plants
16	Genistein	Phytoestrogen	$C_{15}H_{10}O_5$	270.241	*Genista tinctoria*
17	Hesperetin	Flavonoid	$C_{16}H_{14}O_6$	302.27	Lemons and oranges
18	Hippeastrine	Alkaloids	$C_{17}H_{17}$	315.321	*Hippeastrum species*
19	Hordenine	alkaloid	$C_{10}H_{15}NO$	165.24	Barley
20	Hypericin	Naphthodianthrone	$C_{30}H_{16}O_8$	504.45	*Saint John's Wort*

21	Hyperoside	Flavonoid	$C_{21}H_{20}O_{12}$	464.38	*Hypericum perforatum L*
22	Indole-3-carbinol	Glucosinolates	C_9H_9NO	147.18	Vegetables (cabbage)
23	Isoquercitrin	Flavonoid	$C_{21}H_{20}O_{12}$	464.38	Mangoes
24	Kaempferol	Flavonoid	$C_{15}H_{10}O_6$	286.23	*Plants and plant-derived foods*
25	Levodopa	Amino acid	$C_9H_{11}NO_4$	197.1879	*Mucuna Pruriens*
26	Limonene	Terpene	$C_{10}H_{16}$	136.24	Lemon
27	Lutein	Carotenoids	$C_{40}H_{56}O_2$	568.871	Marigold Flower
28	Luteolin	Flavonoid	$C_{15}H_{10}O_6$	286.24	*Celery*
29	Lycopene	Carotenoid	$C_{40}H_{56}$	536.89	Tomatoes
30	Lycorine	Alkaloid	$C_{16}H_{17}NO_4$	287.32	*Lycoris*
31	Mollugin	Quinone	C17H16O4	284.31	*Rubia Cardifolia*
32	Morin	Flavonoid	$C_{15}H_{10}O_7$	302.2357	*Maclura pomifera*
33	Myricetin	Flavonoid	$C_{15}H_{10}O_8$	318.2351	Fruits, berries
34	Naringenin	Flavonoid	$C_{15}H_{12}O_5$	272.257	Grapefruit.
35	p-Coumaric acid	Organic compound	$C_9H_8O_3$	164.16	*Gnetum cleistostachyum*
36	Physostigmine	Alkaloid	$C_{15}H_{21}N_3O_2$	275.346	Calabar bean.
37	Phytic acid	Saturated cyclic acid	$C_6H_{18}O_{24}P_6$	660.04	Cereals and grains
38	Pregnenolone	Steroid hormone	$C_{21}H_{32}O_2$	316.4776	Humans
39	Puerarin	Flavonoid	$C_{21}H_{20}O_9$	416.38	*Radix puerariae*
40	Resveratrol	Stilbenoid	$C_{14}H_{12}O_3$	228.25	*Blueberries*
41	Rosmarinic acid	Polyphenolic compound	$C_{18}H_{16}O_8$	360.32	*Rosmarinus officinalis*
42	Silibinin	Flavonoid	$C_{25}H_{22}O_{10}$	482.44	Milk thistle seeds
43	Tazettine	Alkaloid	$C_{18}H_{21}NO_5$	331.363	*Galanthus transcaucasicus*
44	Tetramethylpyrazine (TMP)	Alkaloids	$C_8H_{12}N_2$	136.2	*Ligusticum wallichii Franch*
45	Vinpocetine	Alkaloid	$C_{22}H_{26}N_2O_2$	350.454	*Vinca minor*
46	Wogonin	Flavonoid	$C_{16}H_{12}O_5$	284.27	*Scutellaria baicalensis*
47	Xanthorrhizol	Sesquiterpenoid	$C_{15}H_{22}O$	218.3	*Cucuma xanthorrhiza*

3.3.1 Chemical diversity of the short-listed compounds:

Natural compounds of different chemical diversity were short-listed based on the literature evidence of their application in neurological diseases. The compounds short-listed were belonged to the group of Phenolic compounds, Flavonoid, Stilbenoid,

Vanilloids, alkaloids, Quinones, Phytoestrogens, Organic compounds, Alkaloids, Steroids, amino acids, Carotenoids, Glucosinolates, and are shown in Figure 8.

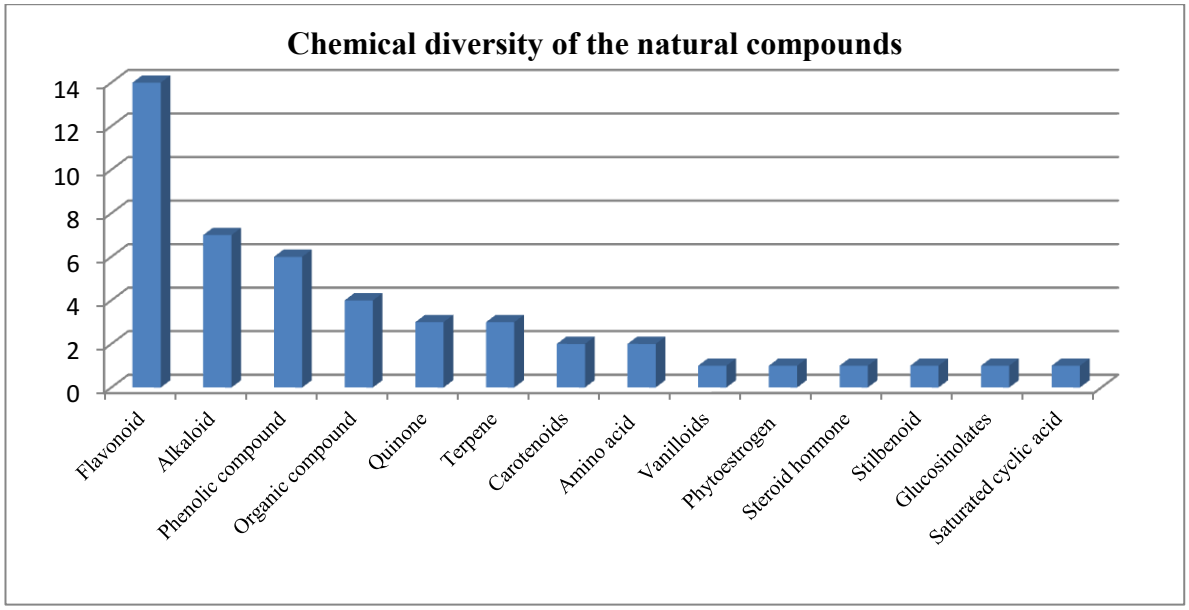

Figure 8. Chemical diversity of short-listed compounds.

3.4 Summary:

The chapter-3 gives the summary of available scientific and traditional information on natural compounds with potential therapeutic applications in neurological diseases. Phytochemicals and respective derivatives were short-listed based on scientific evidence of their application in the treatment of neurological diseases. A total of 47 phytochemicals with neuroprotective and memory enhancing properties through diverse mode of action including antioxidant and anti-inflammatory properties, acetyl choline esterase inhibitory activity, prevention of Aβ formation and deposition, BACE1 enzyme inhibitor etc are short-listed. The compounds short-listed were belonged to the group of Flavonoid (14 compounds), Phenolic compounds (6 compounds), Stilbenoid (1 compound), Vanilloids (1 compound), alkaloids (7 compounds), Quinones (3 compounds), Terpenes (3 compounds), Phytoestrogen (1 compound), Organic compounds (4 compounds), Steroids (1 compound), amino acids (1 compound), Carotenoids (2 compounds), saturated cyclic acids (1 compound) and Glucosinolates (1 compound).

The short-listed phytochemicals and their derivatives were further screened for BACE1 inhibitory activity using *in silico* tools and are detailed in Chapter-4.

Chapter-4

4. *In silico* screening of natural compounds:

4.1 Introduction:

Drug discovery and development is a very expensive, laborious and time consuming process which costs around 1 to 1.8 billion in total per drug (Morgan *et al.,* 2011; Earm and Earm, 2014) and takes around 10 to 15 years of time for the development (Silverman and Holladay, 2014; Winegarden, 2015). Drug molecule has to pass through various stages of drug development such as preclinical and clinical stage, and regulatory approval before it is made available for human application. During the development, drug molecules have very high attrition rate and the main causes are identified to be pharmacokinetics (39%), efficacy (30%) or toxicity (11%) (Wang and Urban, 2004). The cost of the drug development increases with the drug attrition rate during its development (Paul *et al.,* 2010) and hence main focus given on predicting the pharmacokinetic , biological activity and toxicity profile of drug molecule at early stage of drug development.

At this juncture, virtual screening dramatically changed the course of drug discovery process (Boruah *et al.,* 2013). Screening of compounds having high affinity for biological targets and biologically active compounds from large database is called as virtual screening or *in silico* screening (Ekins *et al.,* 2007; Prakhov *et al.,* 2010). Computational tools such as AutoDock, Glide (Chen *et al*., 2012; Lagunin *et al.,* 2014) and ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) tools are used widely for studying molecular interactions of ligands with target and ADMET properties to predict the drug like properties of a compound. *In silico* screening gives early indication of drug failure and hence can reduce the time and cost of drug development (Gad, 2008; Modi *et al.,* 2012).

Virtual screening procedures including molecular docking and ADMET studies are recently developed as an alternative to the *in vitro* high throughput screening (HTS) approaches, which are otherwise highly expensive, time consuming and laborious.

Some of the commonly used virtual screening tools are listed in Table 5 (Heal, 2003). Virtual screening using these *in silico* tools saves a lot of time, cost and provides guidance for selection of molecules for further studies. However, further confirmation of *in silico* predictions using appropriate *in vitro* and *in vivo* methods are always recommended. This is necessary due to limitations of *in silico* tools such as precision of the results (Gleeson *et al.,* 2012).

Table 5. List of virtual screening tools (Heal, 2003).

Sl. No	**Tool**	**Vendor**
1	AutoDock	Accelrys, Inc
2	Gold	Cambridge Crystallographic Data Centre
3	DOCK	University of California at San Francisco
4	FlexX	Tripos, Inc
5	Glide	Schrodinger, Inc
6	ICM	Molsoft, Inc
7	LigandFit	Accelrys, Inc

Molecular docking tools analyze the possible binding, spontaneous affinity and energy requirement for the interaction of particular ligand and target (Ekins *et al.,* 2007). Docking score and docking energy obtained as an outcome of docking studies are indicative of molecular interaction properties. The structures of synthetic and natural compounds available in several databases worldwide were utilized for docking study and generally, 3D structures of the receptors are collected from Protein Data bank (PDB) (Chen *et al*., 2012; Boruah *et al.,* 2013; Lagunin *et al.,* 2014). In general, molecular docking studies are utilized in pharmaceutical drug development, screening and short-listing of large set of compounds for *in vitro* and *in vivo* studies.

The selected lead compound from virtual screening should have certain physiological properties such as drug likeness, potential absorption, distribution, metabolism, and excretion and safety profiles favorable for drug development. Screening all these parameters for all the ligands using *in vitro* and *in vivo* techniques will incur lot of time, cost and are laborious. ADME parameters and possible mode of screening are illustrated in Figure 9 (Pelkonen *et al*., 2011).

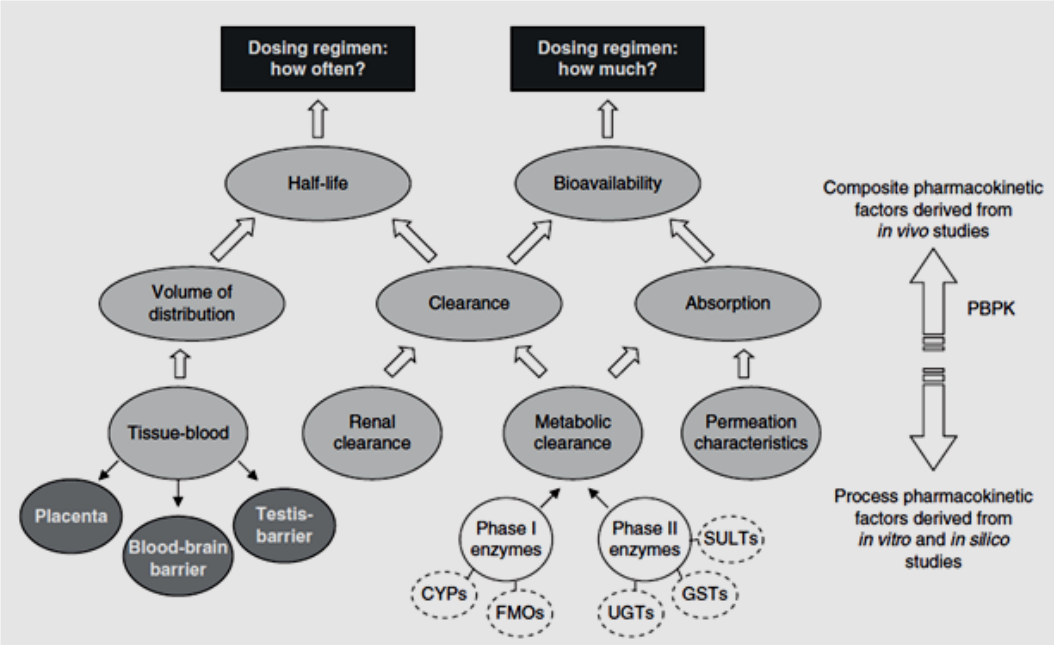

Figure 9. Clinically important pharmacokinetic factors and the most important underlying process or mechanistic pharmacokinetic factors. CYP=cytochrome P450; FMO=Flavin-containing monooxygenase; GST=glutathione-S-transferase; PBPK=physiologically pharmacokinetics; SULT=sulfotransferase; UGT=uridine diphosphoglucuronosyltransferase (Source: Pelkonen *et al*., 2011)

In spite technical advancements, drug development for the treatment of Alzheimer's disease involves many additional challenges and the rate of drug failure was found to be 99.6% (Cummings *et al.,* 2014). There are many reasons for drug failure during the translation of lab success into the application in human (Langley, 2014). These problems were reduced to an extent by the use of different *in silico* tools (Langley, 2014). *In silico* tools are developed based on gained knowledge in *in vitro* and *in vivo* studies. It is now realized that *in silico* methods would be appropriate for avoiding the drug failures observed in animal model based results through modeling, prediction, virtual screening and ADMET analysis (Wall and Shani, 2008).

On the whole, the application of *in silico* evaluation during drug discovery and development could drastically reduce the cost associated with screening large number of compounds and also the cost associated with drug failure in preclinical and clinical studies.

4.2 Research Work:

As discussed in the previous sections, BACE1 is one of the recently emerging potential targets for screening anti- Alzheimer's disease compounds for developing drugs (Gosh *et al*., 2012). The chapter aims at identifying a potential natural ligand for BACE1 and also to evaluate the potential drug likeliness and ADMET properties for selected compounds. Keeping these in mind, the *in silico* screening studies were carried out as discussed below:

(i) DOCKING: *In silico* screening of molecular binding efficiency of selected natural compounds with chosen target BACE1 using AutoDock.

(ii) ADMET Prediction: Predicting and analyzing *in silico* molecular physico-chemical properties, drug-likeliness and ADMET of selected natural compounds.

(iii) Short-listing compounds for *in vitro* studies based on docking and ADMET studies.

4.3 Materials and Methods:

a. Docking studies:

Selection of PDB file for BACE1: An X-ray crystallographic structure of BACE1 with 2.60 Å resolution was selected based on resolution, source, absence of mutation and cited literature. The PDB file 2QU2.pdb (www.rcsb.org) was selected for this study based on the above mentioned criteria.

Molecular docking Software: AutoDock 4.0, noncommercial automated docking software was used in this study. AutoDock is commonly used docking software for predicting interaction with target protein and commonly used in virtual screening of compounds in drug discovery.

Compounds: Natural molecules which had proven neurobiological activities were selected for the purpose of screening against BACE1 as detailed in Chapter 3 of this thesis.

Procedure: The binding affinity of short-listed chemicals with BACE1 was evaluated *in silico* by Molecular docking studies. Docking studies were carried out by using the *in silico* tool, AUTODOCK 4, according to the specified instructions, where ligand in an arbitrary conformation and orientation was used to find its favorable dockings in a protein binding site. The typical docking procedure is shown in Figure 10.

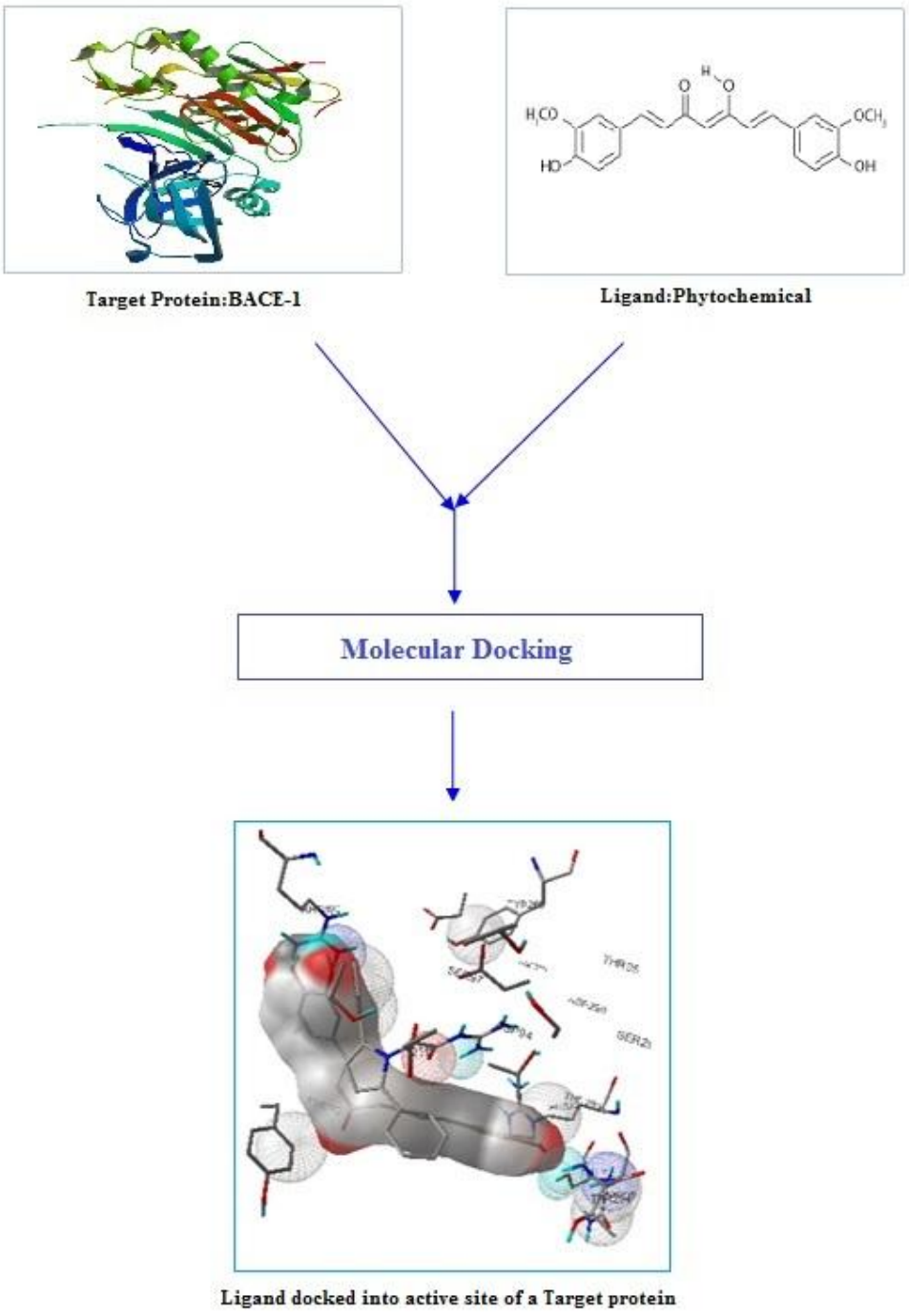

Figure 10: Pictorial representation of docking procedure (Source; www.rcsb.org)

b. Drug like Properties:

ADMET Prediction of BMC and Curcumin were carried out using online tools such as MOLSOFT and Osiris.

Chemical properties of BMC and Curcumin such as molecular formula, molecular weight, number of hydrogen bond acceptor (HBA), number of hydrogen bond donor (HBD), octanol/water partition coefficient (molLogP), water solubility (molLogS), Polar Surface Area (molPSA), volume, drug-likeness score were calculated using MOLSOFT. Drug like properties were analyzed using online ADMET tool called Osiris.

'Drug likeness' is a complex measure of various molecular properties and structure features of ligands to determine its properties for qualifying as a drug candidate. Drug-like properties was first established by Lipinski (Lipinski's Rule of Five) to evaluate drug likeness of investigation drugs early in the development stage. As per the Lipinski's rule, five key physiochemical properties are required to be met to predict BBB permeability of the compound by passive diffusion (Mikitsh *et al*., 2014). This rule is based on the molecular properties of the compound which can predict the drug's pharmacokinetics in human body. Initially, the rule was limited to LogP, Molecular weight, polar surface area, and number of hydrogen bond acceptors and donors. But later, it has been extended to other properties as listed in Table 6. The attributions of successful CNS drug are listed in Table 7.

Table 6: Criteria for Drug-likeness (Mikitsh *et al*., 2014; Pajouhesh *et al*., 2005).

Compound	MW	Rotational bonds	H-acceptors	H-donors	LogP value	LogS	Polar surface area
Ideal drug Characteristics	≤500	≤10	≤10	≤5	≤5	≤-5	≤120 A^2

Table 7: Attributions of a successful CNS drug (Mikitsh *et al*., 2014; Pajouhesh *et al*., 2005).

Compound	MW	Rotational bonds	H-acceptors	H-donors	LogP value	Aqueous Solubility	Polar surface area
CNS drug	≤450	≤8	≤7	≤3	≤5	60µg/ml	≤60-70A^2

Software tools: The tools such as Molsoft and Osiris property explorer were used for determination of the drug likeness of the molecules. Osiris Property explorer has been used as a reference tool, while Molsoft has been used as the focal tool for the analysis.

a) Molsoft uses a fragment based approach for calculating individual properties of a molecule. A molecule is divided into a set of fragments (linear or non-linear) of different length and representation levels. It is then counted for the number of occurrences of each chemical pattern found against the in house reference database.

The drug like score in Molsoft is calculated by a fragment based method, wherein the molecule's substructures are compared with a database having fragments of known approved drug as well as non-drug fragments. As per molecular property explorer, 80% of the marketed drugs were found to have positive value for drug-likeness.

b) Osiris property explorer uses an increment system for calculating the individual properties; adding contributions of every atom based on its atom type. This is based on more than 5,000 compounds with experimentally determined values which are used as a reference set.

In Osiris Property explorer, the drug score is calculated by combining molecular properties such as drug likeness, Log P, log S, molecular weight and toxicity risks in one value to judge the compound's overall potential as a drug (http://www.organic-chemistry .org / prog/peo /drugScore .html). As per the database properties user manual, a drug score is a real value ranging from 0 to 1. A score of 1 indicates that a compound is a good candidate to be a drug, whereas a score of 0 indicates that a compound is not a good candidate to be a drug.

4.4 Results:

4.4.1. *In silico* Docking studies:

The structure of BACE1 from pdb file and the picture showing docking of BMC with BACE1 are shown in Figure 11 and Figure 12. The results of docking studies carried out using Autodock.4 are shown in Table 8.

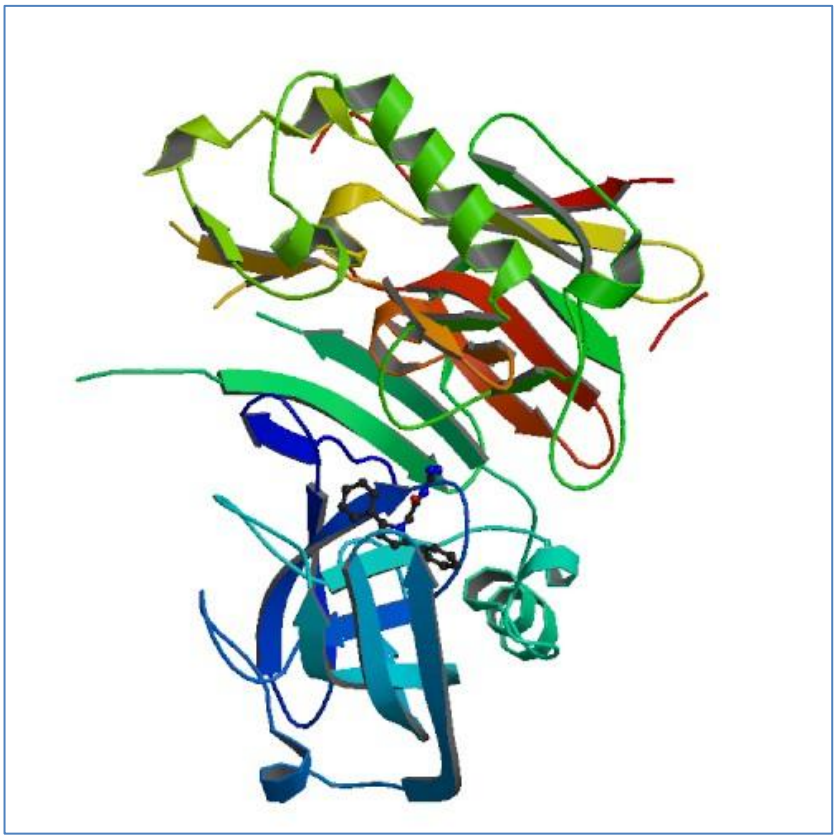

Figure 11: Structure of BACE1 from PDB file (www.rcsb.org)

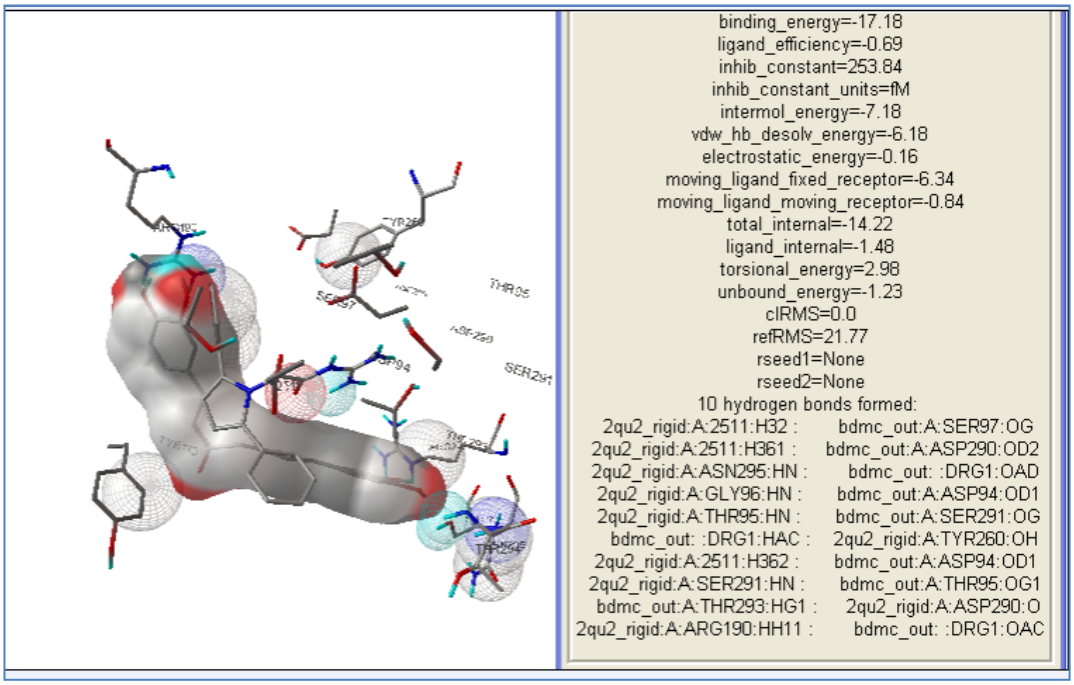

Figure 12: Docking output of BMC with BACE1

Table 8: Results of molecular docking study; Binding energy for phytochemicals

Sl. No	Name of compound	Binding Energy (kcal/mol)
1	6-hydroxyflavone	-17.38
2	Acetyl-L-Carnitine	-16.12
3	Allicin	-15.96
4	Asthaxanthin	-14.94
5	Bis-demethyl curcumin (BMC)	-17.18
6	Capsaicin	-15.32
7	Catechin	-18.15
8	Chlorogenic acid	-15.85
9	Cinnamic acid	-17.75
10	Coumaric acid	-16.61
11	Creatine	-16.77
12	Curcumin	-14.33
13	Ellagic acid	-18.51
14	Emodin	-18.95
15	Ferulic Acid	-16.61
16	Fisetin	-18.63
17	Genistein	-18.25
18	Hesperetin	-17.23
19	Hippeastrine	-17.29
20	Hordenine	-15.98
21	Hypericin	-21.41
22	Hyperoside	-14.69
23	Indole-3-carbinol	-15.57
24	Isoquercitrin	-15.25
25	Kaempferol	-19.42
26	Levodopa	-17.27
27	Limonene	-15.33
28	Lutein	-17.66
29	Luteolin	-19.97
30	Lycopene	-13.67
31	Lycorine	-17.04
32	Mollugin	-17.92
33	Morin	-19.01
34	Myricetin	-13.65
35	Naringenin	-18.89
36	Physostigmine	-17.47
37	Phytic acid	-13.56
38	Pregnenolone	-18.61

39	Puerarin	-16.12
40	Resveratrol	-16.61
41	Rosmarinic acid	-15.82
42	Silibinin	-16.3
43	Tazettine	-18.18
44	Tetramethylpyrazine	-5.16
45	Vinpocetine	-16.99
46	Wogonin	-19.18
47	Xanthorrhizol	-15.31

BMC, a metabolite of widely studied phytochemical Curcumin in the prevention and treatment of Alzheimer's disease was selected for further studies. Curcumin was used as a reference molecule in analyzing drug like properties and in *in vitro* studies. Other phytochemicals considered in the docking study are either already studied for application in Alzheimer's disease or are in development stages and hence were not considered for further studies.

4.4.2 Drug Like Properties:

The molecular properties of BMC and Curcumin were studied in molecular property explorer viz Molsoft and Osiris. Chemical properties of BMC and Curcumin such as molecular weight, molecular formula , number of hydrogen bond acceptor (HBA), number of hydrogen bond donor (HBD), octanol/water partition coefficient (molLogP), water solubility (molLogS), polar surface area (molPSA), volume and drug likeness score were calculated using MOLSOFT and the results are shown in Table 9. Drug properties were evaluated using Osiris and the results are shown in Table 10.

Table 9: Results from molecular property evaluation using Molsoft

Sl. No	Molecular Properties	BMC	Curcumin
1.	Molecular Formula	C_{19} H_{16} O_6	$C_{21}H_{20}O_6$
2.	Molecular Weight	340.09	368.13
3.	Number of HBA	6	6
4.	Number of HBD	4	2

5.	molLogP	2.44	3.08
6.	molLogS	-4.02 (in Log (moles/L)) 32.44 (in mg/L)	-4.35(in Log (moles/L)) 16.27 (in mg/L)
7.	molPSA	94.97 A^2	76.84 A^2
8.	molVol	351.46 A^3	392.61A^3
9.	Rotational Bonds	10	8
10.	Drug likeness Score	-0.82	0.35
11	Violation of Rule of 5	0	0

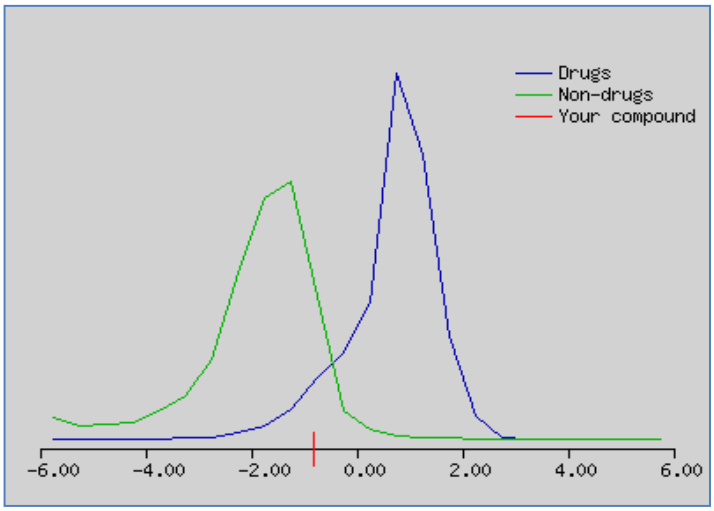

Figure 13: Drug likeness score graph for BMC (score: -82)

Table 10: Results from molecular property evaluation using Osiris property explorer

Sl. No	**Molecular Properties**	**BMC**	**Curcumin**
1.	Molecular Weight (g/mol)	340.00	368.38
2.	cLogP	2.59	2.97
3.	LogS (Aqueous Solubility)	-2.99	-3.62
4.	Drug likeness	-3.48	-3.95
5.	Drug score	0.43	0.39

From the results, it can be concluded that BMC possesses better CNS drugs features than curcumin. Fundamental features of CNS (central nervous system) drugs are related to their ability to cross the BBB and exhibit CNS activity. Assessment of drug for its ability of cross BBB is very important in CNS drug discovery and development. The Blood Brain Barrier (BBB) is an unique membrane that segregates brain from circulating blood via tight junctions. The tights junction's results in very high trans-endothelial electrical resistance compared to other tissues, reducing the aqueous based paracellular diffusion (Mahmoud *et al.,* 2003). The micro vessels present in the BBB are principle route of chemical transport across the BBB. Apart from this, endothelial cells in the BBB also contain several degradative enzymes and P-glycoproteins (Pgp), active drug efflux transporters (Mishra *et al*., 2003)

i. **LogP value :** LogP (octanol/water partition coefficient) is the measure of lipophilicity of the given drug. Higher lipophilicity is linked to poor solubility and lower bioavailability. LogP value for both the compounds was less than 5 and hence meets the LogP criteria for CNS drug as cited above. BMC has a LogP which is comparatively less than Curcumin. Based on LogP it can be said that BMC could be absorbed more easily than Curcumin and the CNS penetration could also be higher if we see solely in terms of LogP. As per the study conducted by Hansch and Leo, compound with LogP value ranging 1.5 to 2.7 will have optimal blood brain barrier penetration, which applies to BMC (Pajouhesh *et al*., 2005).

ii. **Polar surface area:** Polar surface area (PSA) of a molecule is the surface belonging to polar atoms and is a descriptor which can predict transport properties of drugs across the cell membrane. A drug like molecule should not possess a PSA exceeding $120A^2$ with the optimal range being 90-120 A^2 (Ertl *et al*., 2000; Blake, 2000). For CNS drugs it has been estimated that the PSA is at 60-$70A^2$ (Kelder., 1999; Waterbeemd., 1998). A 2003 study conducted has seen that the most of the CNS drugs in phase II trials lie in the range of 30-$50A^2$. In a study conducted by Kelder, it was concluded that the cutoff for CNS penetration is $90A^2$ (Kelder., 1999; Waterbeemd., 1998).

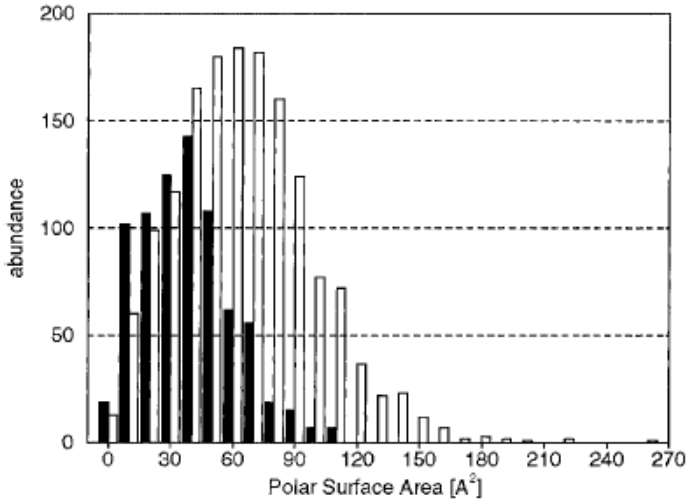

Figure 14. Polar surface area for Central Nerves System (CNS) drugs: Dark colored bars; CNS drugs, Light colored bars; Non-CNS drugs (Source; Waterbeemd, 1998; Kelder, 1999)

Both BMC and Curcumin seem to be on the higher side with BMC (94.97 A^2) having PSA more than that of Curcumin (76.84 A^2). PSA can also be indirectly related to the hydrogen bond donors (Nitrogen (N) and oxygen (O)). The number of donors is higher for BMC (4 Hydrogen donor's) than curcumin (2 Hydrogen donor's) and hence probably higher PSA. It is also known that, the lead compound will have a higher probability of crossing the BBB, if the sum of the nitrogen and oxygen atoms in the compound is five or less (Pajouhesh *et al*., 2005).

iii. **Molecular weight**: Molecular weight plays an important role for drug candidates targeting the Blood brain barrier (BBB). BBB is formed of tight junction between endothelial cells in CNS vessels separating the circulating blood and cerebrospinal fluid. The compounds with a molecular weight less than 500 Da can cross the BBB with simple diffusion (Mikitsh *et al*., 2014). But according to statistical estimates the drugs in market, which crosses the BBB have a mean molecular weight of 310 g/mol (Waterbeemd *et al.,* 1998)

BMC has a molecular weight less than that of Curcumin (340 as against 368.38 g/mol), and hence a higher probability of BBB penetration.

iv. **Solubility:** Aqueous solubility of drug is very important as it affects the bioavailability of the drug. Normally, poor aqueous solubility leads to poor

bioavailability. However, this can be addressed during the formulation development using various technologies available for improving the drug solubility and bioavailability. Nearly 80% of the drugs have LogS value greater than -4 (http://www.organic-chemistry.org/prog/peo/logS.html).

Based on the Molsoft, BMC is shown to have solubility of 32.44 mg/L, where as Curcumin was shown to have 16.27 mg/L in water. Similarly from Osiris results, BMC was shown to have LogS value of -2.99 mg/L, whereas Curcumin was shown to have LogS value of -3.45. The LogS values for both BMC and Curcumin are greater than -4, indicating that the BMC can be a good drug candidate (http://www.organic-chemistry.org/prog/peo/logS.html).

v. **Hydrogen Bonds:** The total number of hydrogen bonds formed (acceptors and donors) were 10 for BMC and 8 for Curcumin. BMC was shown to interact with SER 97, ASP290, DRG1, ASP94, SER291, TYR 260, ASP 94, THR 95, ASP290 and DRG1 residues in the active site of BACE1.

This could be viewed in 2 ways. Higher hydrogen bond formation can lead to greater stability in binding to the target. Also, a higher hydrogen bonding capacity is negatively correlated to the uptake in CNS with <5 H-bond donors and <10 H-bond acceptors being acceptable for a CNS drug candidate (Mikitsh *et al*., 2014). It has been seen that small molecules having a high hydrogen bonding capacity have a minimal distribution across the CNS (Pardridge and Mietus, 1979; Leeson and Davis, 2004). Both BMC meets the above cited criteria and hence could be a potential CNS drug candidate.

vi. **Rotational Bonds:** This parameter is a measure of molecular flexibility by which the compound transverses cell membrane (Pajouhesh *et al*., 2005) and can predict oral bioavailability of drugs. Rotatable bond is a non-ring single bond, bounded to non-hydrogen atom.

vii. (http://www.molinspiration.com/services/properties.html). Most of CNS drugs will have 5 or <5 rotational bonds (Pajouhesh *et al*., 2005)

BMC has a higher number of rotational bonds than curcumin (10 against 8). Higher rotational bonds will lead to higher flexibility of the molecule but it might lead to non-specific binding. Curcumin is nontoxic and since BMC is a demethylated form of curcumin, it can be expected that the higher number of rotational bonds should not be a concern.

viii. **Drug likeness score and Drug score:** As explained earlier, the drug likeness score is a comparative study, calculated based on a fragment based approach with a reference database of molecules. The drug score is the combination of the individual molecular properties of the compound. The drug score for BMC is 0.43 and Curcumin is 0.39. The drug likeness score for BMC is -0.82 and Curcumin is 0.35.

Though the drug likeness score is less for BMC, the drug score is marginally higher than Curcumin. Also, taking into consideration the individual properties, BMC seems to be favorable in terms of the important criteria for optimum ADME properties such as LogP, solubility and Molecular weight.

4.5 Summary:

In this research study, several tested phytochemicals showed varied binding energy against BACE1, ranging from -5.16 to -21.41kcal/mol. However, BMC, a natural metabolite of curcumin, widely studied in the treatment of Alzheimer's disease was considered for further studies. Multiple pathways are suggested for Alzheimer's disease, which led to several drug targets in the treatment of Alzheimer's disease, thus necessitating the need of multi-target drugs, which can interfere in several pathways to treat Alzheimer's disease without causing any side effects. It is unlikely that, a drug acting on single target will be helpful in treating Alzheimer's disease. Multi-target drugs are often phytochemicals (Russo *et al.,* 2013), which are known for higher safety profile. Curcumin is a well known natural compound to have diverse biological activity with minimal or no toxicity in several studies on humans. It is known to have multiple beneficial effects such as prevention of Aβ plaque formation, delayed degradation of neurons, metal-chelation, anti-inflammatory activity, antioxidant activity and decreased microglia formation, which improves cognitive functioning in Alzheimer's disease patients (Mishra and Palanivelu, 2008). Though, till date systematic structure and

activity relationship has not been established, research interest continues to explore the diverse biological activities of curcumin analogues and its metabolites. Curcumin and curcumin metabolites possess diverse biological activities having multitarget potency. Among short-listed natural compounds, Resveratrol and Curcumin can bind to many drug targets of human diseases (Ji *et al.,* 2009) showing their multitarget potency. Resveratrol had less binding energy of -16.67, when compared to BMC, a metabolite of curcumin (-17.18) indicating its higher affinity towards BACE1. With the similar interest, we made an effort to evaluate the multi-target potency of one of the natural metabolite of Curcumin, BMC, in order to develop it as an effective therapeutic option for treatment of Alzheimer's disease. Curcumin was also evaluated along with BMC as a reference molecule *in silico* (molecular properties) and *in vitro* studies.

BMC showed the binding energy of -17.18 kcal/mol, whereas curcumin showed the binding energy of -14.33 kcal/mol, indicating strong affinity of BMC for BACE1. Both BMC and curcumin were subjected to the evaluation of drug-likeness. In the further drug like property studies (molsoft), BMC was found to have drug likeliness score of -0.82, which is less than the score indicated for a good drug candidate, whereas Curcumin was found to have positive score of 0.32. However, BMC (0.43) had a higher drug score than that of Curcumin (0.39), which meets criteria of being a good drug candidate, indicating characteristic of a potential drug candidate in the therapeutic intervention for Alzheimer's disease. Further, BMC and Curcumin were studied in *in vitro* studies.

Chapter-5

5 *In vitro* evaluation of BMC and Curcumin

5.1 Introduction:

In vitro studies have played an indispensable role to validating the *in silico* results for translating it towards a successful drug therapy. Even though *in silico* methods are widely used in modern drug discovery process for initial screening of compounds, still screened compounds need to be validated in *in vitro* to confirm the *in silico* predictions. Strategy of combining *in silico* and *in vitro* methods for efficient drug discovery is demand of the hour to reduce cost and improve drug discovery effectiveness (Pauli *et al.,* 2008).

Having limited understanding of underlying Alzheimer's disease mechanism, several different treatment strategies were adopted which includes, inhibition of many receptors and enzymes such as acetylcholinesterase and α, β & γ – secretases. Apart from these, other auxiliary physiological events such as oxidative stress and inflammatory immune responses also play a major role and hence, molecules with antioxidant and anti-inflammatory properties may be an ideal choice for therapeutic drug development as it may delay the progression of Alzheimer's disease. There are many studies that support pathophysiology of Alzheimer's disease due to induced oxidative stress (Thies *et al*., 2013). Oxidative stress is considered as one of the several factors involved in the onset of Alzheimer's disease and is well associated with reduced antioxidant activity in affected Alzheimer's disease patients (Hebert *et al*., 2003). Reactive oxygen species (ROS) are generated naturally in humans as part of normal human metabolism and increases in levels during aging, infection and stress. Antioxidant enzymes responsible for antioxidant activity including glutathione peroxidase and superoxide dismutase, helps to reduce ROS and manage oxidative stress. In Alzheimer's disease patients levels of these enzymes were found to be less and this lead to oxidative stress and subsequently oxidative damage in Alzheimer's disease patients. Highly reactive chemical species including ROS, superoxide radicals, peroxides, nitric oxide species, and physiological imbalance were identified as major cause of oxidative stress induced

disorders and diseases. In Alzheimer's disease patients, ROS accumulation can surpass both enzymatic and non-enzymatic antioxidants, causing DNA, lipid and protein oxidation thus becoming responsible for neuroapoptosis (Roberts *et al*., 2009). Oxidative stress induced dementia was sometimes related to the increased accumulation of redox active sources, such as transition metal ions (copper, iron and zinc) during the early stages of Alzheimer's disease (Daborg *et al*., 2013), which potentially bind to the Aβ that in turn initiating subsequent cellular physiology. The metabolic consequences of oxidative stress in the onset of Alzheimer's disease are shown in Fig. 15. Oxidative stress can cause serious damages to cell and its metabolism that leads to a variety of human diseases including Alzheimer's disease (Mogana *e al.,* 2013). Thus, compounds with high anti-oxidant potential can be a good choice of drug to delay the progression of Alzheimer's disease. Hence, *in vitro* screening of compounds with potent antioxidant activity is one of the well adopted strategy to screen and develop therapeutics for Alzheimer's disease. Most commonly used anti-oxidant screening methods include ABTS (2, 2-azinobis (3-ethylbenzothiazoline-6-suifonic acid)) radical cation decolorization assay, DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging assay, Ferric Reducing Anti-oxidant power (FRAP) Assay and superoxide scavenging assay.

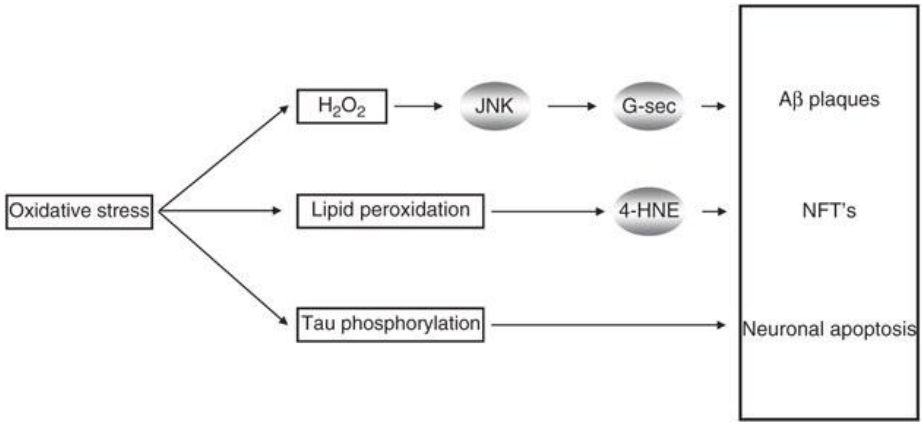

Figure 15. Oxidative stress in the pathophysiology of Alzheimer's disease (Source; Manolopoulos *et al*., 2009).

Inflammation is another important consequence of oxidative stress and Aβ plaques. Inflammation is mediated by two enzymes namely, the cyclooxygenase (COX) and 5-lipoxygenase (LOX) by producing prostaglandins and leukotrienes respectively from arachidonic acid (Mogana *et al.,* 2013). In addition to these pathways, overproduction of free radical nitric oxide from inducible nitric oxide synthase (iNOS) also elicits

leukotrienes and cytokines (Soneja *et al*., 2005; Russell *et al*., 2011) and hence adds to inflammation. Higher level of tissue pro-inflammatory cytokines such as interleukins-1 β (IL-1β), tumor necrosis factor α (TNFα), and interferon γ (IFNγ) were found to increase the Aβ peptide and tau phosphorylation (Joshi *et al*., 2015; Dyall, 2010). IL-1 induces the oxidative stress causing lipid peroxidation, activates microglial cells to produce inflammatory cytokines and increases the synthesis of APP (Dyall, 2010). In this view, the inhibition of LOX by a compound will be considered as anti-inflammatory compound with therapeutic interest. Hence, *in vitro* determination of inhibition of proinflammatory cytokines and LOX enzyme inhibition were utilized in the present study to evaluate anti-inflammatory potential of the short-listed compounds.

BACE1 is an important and attractive drug target for the development of drug for the treatment of Alzheimer's disease. Since the discovery, lot of research work supported the role of BACE1 in the pathophysiology of Alzheimer's disease (Chang *et al*., 2004). BACE1 is involved in the amyloid cascade, which cleaves APP to form Aβ. APP cleavage by BACE1 is the rate limiting step in the formation of Aβ, and hence it is considered as an important therapeutic target in the development of drug for the treatment of Alzheimer's disease (Chang *et al*., 2004; Chami *et al.,* 2012). The development of BACE1 inhibitors was actively perused for several years (Ghosh *et al.,* 2008). Although, the BACE1 binding efficiency was evaluated in *in silico* studies, confirmation through *in vitro* studies is very important.

In the present study, BACE1 fluorescence resonance energy transfer (FRET) assay was used to measure BACE1 inhibitory activity of selected compounds. This assay mainly utilizes the principle of determining a fluorescence emitting due to the enzymatic cleavage of FRET protease substrate by BACE1 enzyme. Inhibition of fluorescence is then correlated with BACE1 inhibitory activity.

5.1.1 Compounds selected for *in vitro* evaluation:

BMC and Curcumin were short-listed from *in silico* study for *in vitro* evaluation.

Curcumin is well known to have diverse biological activity without any toxicity in several human studies. It is well known to have beneficial effects through the prevention

of Aβ plaque formation, delayed degradation of neurons, metal-chelation, anti-inflammatory activity, antioxidant activity and decreased microglia formation, which improves cognitive functioning in Alzheimer's disease patients (Mishra and Palanivelu, 2008). Commercially available Curcuminoids contain 77% Curcumin (Curcumin-1), 17% demethoxycurcumin (Curcumin-II), and 3% bisdemethoxycurcumin (Curcumin-III). Demethoxycurcumin and bisdemethoxycurcumin which differ from Curcumin chemically with methoxy substitution, exhibit significantly different antioxidant, antitumor, and anti-inflammatory activities (Anand *et al*., 2008). Though, till date systematic structure and activity relationship has not been established, research interest continues to explore the diverse biological activities of curcumin analogues and its metabolites. With similar interest, we made an effort to evaluate the multi-target potency of one of the natural metabolite of Curcumin, BMC, in order to develop it as an effective therapeutic option for the treatment of Alzheimer's disease.

Thus, this study aims at evaluating antioxidant, anti-inflammatory and BACE1 inhibitory activity of BMC in comparison with curcumin in anticipation that BMC may be a better therapeutic option compared to Curcumin in management of Alzheimer's disease.

5.2 Research Work:

The present chapter work aims at evaluating *in vitro* efficacy of compounds which were short-listed in *in silico* study:

(i) Measuring the free radical scavenging activity using ABTS (2, 2-azinobis (3-ethylbenzothiazoline-6-suifonic acid)) radical cation decolorization assay

(ii) Measuring the free radical scavenging activity using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method

(iii) Measuring the free radical scavenging activity using Ferric Reducing Anti-oxidant power (FRAP) assay

(iv) Measuring the free radical scavenging activity using superoxide scavenging assay

(v) Evaluating 5-Lipoxygenase (LOX) inhibition ability of selected compounds by LOX inhibition assay

(vi) Evaluating BACE1 inhibition activity of selected compounds using FRET assay

5.3 Materials and Methods adopted:

5.3.1. Evaluation of Antioxidant Properties:

a. DPPH radical scavenging activity:

DPPH radical scavenging activity was determined by the method of Lamaison *et al*., 1990 which is based on the reduction in optical density (OD) of colored methanolic solution of the DPPH free radical at 517 nm. The reaction mixture was prepared by addition of 20 µl of test items (BMC, Curcumin and vitamin C) and 280 µl of DPPH reagent to reach a final volume of 300 µl and kept for incubation in dark for 50 minutes and then read immediate absorbance at 517 nm using spectrophotomèter and IC_{50} was calculated. Percent inhibition was calculated by comparing absorbance of test substance with that of control. The radical scavenging activity of the test substance was expressed as the 50% inhibitory concentration (IC_{50}) that was measured from the plot drawn between concentrations versus percentage of inhibition.

b. ABTS radical scavenging activity:

2,2' Azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) radical cation was prepared by reacting 5 ml ABTS stock solution with 88 µl Potassium persulfate. The mixture was allowed to stand in dark at room temperature for 12-16 hours before use. Stability of the radical is 2 days under dark conditions. Appropriate dilutions of test items (BMC, Curcumin and Vitamin C) were prepared (Bramati *et al.,* 2003).

Step.1; ABTS radical solution was mixed with methanol to get an absorbance of 0.70-0.80 at 734 nm.

Step. 2; 30 µl of diluted ABTS radical solution and 3 ml of methanol (ABTS reagent) were mixed prior to the addition into the plate.

Step3: The reaction mixture was made by addition of 10 µl test substance and 290 µl of ABTS reagent to reach a final volume of 300 µl and absorbance was

measured at 734 nm using spectrophotometer (X mark Micro plate spectrophotometer (BIO-RAD)).

Scavenging efficacy of the test substances were estimated by comparing absorbencies of the test substances with that of control (Re *et al*., 1999).

c. Superoxide Radical Scavenging activity:

Superoxide radical scavenging activity was determined by Nitro blue tetrazolium (NBT) riboflavin photo reduction method as described by McCord and Fridovich. Different concentrations of 30 µl test items (BMC, Curcumin and Gallic acid), 30 µl of EDTA (60 mM), 30 µl of NBT (500 µM), 30 µl of Riboflavin (20 µM) and180 µl of Phosphate buffer (96.67 mM) were added to a total volume of 300 µl in 96 well plate and the plate was illuminated for 15 min and thereafter the absorbance was measured at 560 nm (McCord and Fridovich, 1969., Anto *et al* ., 1996)

Calculations:

- An IC_{50} value was determined as the concentration that elicits the half maximal response.
- Percentage inhibition (%) of Superoxide Scavenging Activity was calculated by as [(A-B)/A] x 100.
- A – Difference in absorbance of control sample between samples with and without riboflavin.
- B – Difference in absorbance of test sample between samples with and without riboflavin

d. FRAP assay (total antioxidant activity):

The FRAP assay (TPTZ assay) developed by Benzie and Strain (Benzie and Strain, 1996) was employed to measure the total antioxidant activity of the test substances in comparison to Vitamin C. FRAP reagent was prepared by the mixing 10 ml Buffer (300 mM acetate buffer pH 3.6), 1 ml of 2, 4, 6-Tripyridyl-S-Triazine (TPTZ) (10 mM TPTZ solution in 40 mM HCl) and 1 ml of $FeCl_3$ solution (20 mM $FeCl_3$ 6H20 (Ferric Chloride) solution) prior to the addition into the plate.

Step 1: The reaction mixture was made by addition of 10 µl of test substance (BMC, Curcumin and vitamin C) of different concentrations and 290 µl of FRAP reagent to reach a final volume of 300 µl.

Step 2: Absorbance was read at 593nm and then EC_{50} was calculated.

All tests were run in triplicate and mean values were used to calculate EC_{50} values. EC_{50} is defined as concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM ferrous salt.

5.3.2. Assessment of anti-inflammatory properties

a. 5-Lipoxygenase inhibition:

5-Lipoxygenase enzyme inhibitory activity was measured using the method described by Chung *et al.,* 1999, Hung *et al.,* 2011 and DSVGK *et al.,* 2014. The 250 µl assay mixture contains 175 µl of 50 mM Tris-Hcl buffer (which contains 20 µl test sample of different concentrations), 5 µl enzyme, 5 µl Linoleic acid and 65 µl Fox reagent

- 5 µl of 5-Lipoxygenase enzyme was added to 50 mM of 175 µL Tris HCl buffer containing test sample.
- 5µl Linoleic acid (140 µM) in 50 mM Tris HCl buffer was added followed by 20 minutes incubation at 25^{o} C and the total reaction mixture volume was 185 µl
- 65 µl Fox reagent containing H_2SO_4 30 mM, Xylenol orange 100 µM, Iron(II) Sulfate 100 µM, Methanol/Water 9:1 was added to stop the reaction.
- The absorbance was read at 595 nm up to 30 minutes at 25^{o}C in Micro plate spectrophotometer.

The percentage enzyme inhibition was calculated using the formula as mentioned below and subsequently, IC_{50} value was determined

$$\% \text{ inhibition of 5-Lipoxygenase} = \frac{(\text{Absorbance of control} - \text{Absorbance in background}) - (\text{Ab sample} - \text{Ab background}) \times 100\%}{(\text{Absorbance of control} - \text{Absorbance in background})}$$

b. Effect of BMC on Cytokine profiles in Lipopolysaccharide (LPS) induced whole blood.

Curcumin and BMC stocks were made in DMSO (150 µg/ml). The final concentrations of Curcumin and BMC used were 10 µg/ml, 20 µg/ml, 40 µg/ml ,60 µg/ml, 80 µg/ml, 100 µg/ml, 120 µg/ml, 140 µg/ml, 150 µg/ml,175 µg/ml. The treatments were done in duplicates. Heparinized whole blood was pre-treated with compound/control for 1h followed by co-treatment with LPS for 6h. Plasma was collected by centrifugation at 1200 rpm, at 4^{o}C for 5 min. ELISA assay was performed for TNF α, IL-1β and IL-6. ELISA assay was carried out as per manufacturer's (R&D DuoSet ELISA kits) instructions. In each assay sample dilution was optimized to fit the range of analysis. All experiments were repeated thrice (Nerad *et al.*, 1992).

5.3.3. BACE1 inhibition activity.

BACE1 Enzyme Assay Kit (No.2985) was used as per the recommendations of manufacturer for the inhibition assay. Purified baculovirus-expressed BACE1 and a new 'red' FRET peptide substrate based on the "Swedish" mutant were used in the assay.

The principle of the BACE1 FRET assay as per the manufacturer (https://tools.thermofisher.com/content/sfs/manuals/L0724.pdf) is as follows: The peptide substrate is synthesized using two fluorophores, a fluorescent donor [a rhodamine (Rh) derivative] and a proprietary quenching acceptor. The distance between these two groups has been selected so that upon light excitation, the donor (D) fluorescence energy is significantly quenched by the acceptor (A) through a quantum mechanical phenomenon known as resonance energy transfer. Once the fluorophore is separated from the quenching group due to the action of BACE1, it restores the full fluorescence yield of the donor. Thus, a weak fluorescent peptide substrate becomes highly fluorescent upon enzymatic cleavage; the increase in fluorescence is linearly related to the rate of proteolysis.

a. Materials used:

White Microwell plates (384-wells), Molecular device M5e instrument (FRET mode), Pipetting devices Molecular devices-spectra Max M5e, BACE1 Enzyme Assay Kit – Invitrogen # 2985. Material provided with BACE1 Enzyme Assay Kit is shown in the Table 11 and preparations of reagents are shown in Table 12.

Table 11. Material provided with BACE1 Enzyme Assay Kit

Component	Description	Quantity	Part No	Storage before 1st use
BACE1 Enzyme	50 mM Tris (pH 7.5), 10% glycerol	5 Units (~58µl)	P2947	-80ºC
BACE1 Substrate, 75 µM	Rh-EVNLDAEFK-Quencher, in 50 mM Ammonium bicarbonate	45 µl	P2986	-20ºC
BACE1 Stop Solution	2.5 M Sodium acetate	5 ml	P2985	20-30 ºC
BACE1 Assay Buffer	50 mM Sodium acetate (pH 4.5)	20 ml	P2988	20-30 ºC

b. Reagent preparation:

Table 12. Preparation of reagent provided in assay kit

Sl No.	Component	Concentration	Volume taken µl	Assay buffer added µl	Total volume µl	3X concentration
1	BACE1 Enzyme	5 units; 83.5units/ml	3	122	125	2 unit/ml
2	BACE1 Substrate	75 µM	1.25	123.5	125	750 nM
3	BACE1 Product Standard, 25 µM	25 µM	1	32	33	750 nM

BMC sample Preparation:

Accurately 1 mg of BMC (Purity >88.76%) was weighed and 3 mg/ml concentration was prepared by dissolving in appropriate amount of DMSO and the dilutions were done as shown in the Tables 13 and 14.

Table 13. Preparation of BMC samples for assay

BMC (3mg/ml) in µl	DMSO in µl	BMC Concentration in µg/ml
15	35.0	900
12.5	37.5	750
10	40.0	600
7.5	42.5	450
5	45.0	300
2.5	47.5	150
1.25	48.75	75

Table 14. Preparation of BMC 3X stocks in assay buffer

BMC Concentration µg/ml	Volume taken µl	Assay buffer added µl	Total volume µl	Final concentration of BMC µg/ml
900	0.5	24.5	25	18
750	0.5	24.5	25	15
600	0.5	24.5	25	12
450	0.5	24.5	25	9
300	0.5	24.5	25	6
150	0.5	24.5	25	3
75	0.5	24.5	25	1.5
37.5	0.5	24.5	25	0.75
15	0.5	24.5	25	0.3
1.5	0.5	24.5	25	0.03
0.15	0.5	24.5	25	0.003

c. Procedure:

1. 5µl 3X of BACE1 enzyme was added to 5µl 3X test compound (or BACE1 Assay Buffer containing 2% DMSO (Dimethyl sulfoxide) to prepare a control well) and mixed well.
2. To start the reaction, 5µl of 3X BACE1 Substrate was added and mixed well. The fluorescence was monitored at 545 nm excitation and 585 nm emission

settings in FRET mode to record 0 minute reading. At this point, concentration of Enzyme, substrate and test compound is 1X.

3. The reaction mixture was incubated for 120 minutes at room temperature in dark. The fluorescence was tracked either kinetically in real-time manner or every 30 min. for two hours.
4. The fluorescence was monitored at 545 nm excitation and 585 nm emission settings in FRET mode in Molecular device M5e instrument. Volumes of reagents used in the assay were shown in Table 15.

Note: Controls (no inhibitor) were maintained intermittently between test reactions. This was done in order to correct for any increase in fluorescence due to a time delay in reaction initiation between samples.

Table 15. Assay reagents and volumes in the procedure of BACE1 assay

Reagents	**Standard Enzyme Reaction mixture in µl**	**Enzyme + Test Compound in µl**	**Positive control (With BACE1 inhibitor)**
BACE1 Assay Buffer (Containing 2% DMSO)	5	--	--
BACE1 Substrate (750 nM)	5	5	5
Test Compound/ BACE1 inhibitor		5	5
BACE1 Enzyme (2.0 U/ml)	5	5	5
Total Volume	**15 µl**	**15 µl**	**15 µl**

Calculation:

- An IC_{50} value was determined as the concentration that elicits the half maximal response.
- Percentage inhibition (%) was calculated by = 100- (X*100/Y).
- Y -- Fluorescence of control sample
- X – Fluorescence of test sample

5.4. Results:

5.4.1 Evaluation of Antioxidant Properties:

Antioxidant activity of BMC along with Curcumin was carried out using DPPH, ABTS, and superoxide radical scavenging assays including FRAP assay. The results are shown in Figures 16, 17, 18 and 19. Percentage differences in IC_{50} values of Curcumin and Vitamin C in comparison with BMC are shown in Table 16. In *in vitro* studies, BMC was found to have superior antioxidant activity compared to its parent compound Curcumin and reference compound Vitamin C. It was found to have 5.4 times lower IC_{50} value of Curcumin and 4.9 times lower IC_{50} value of Vitamin C in DPPH radical scavenging assay. In ABTS radical scavenging assay, BMC was found to have 4.1 times lower IC_{50} values of both Curcumin and Vitamin C. The IC_{50} value of BMC was 14.7 and 1.1 times lower when compared to IC_{50} values of Curcumin and Vitamin C respectively in superoxide radical scavenging assay. In FRAP assay, it has shown 5.8 times lower IC_{50} value than Curcumin and 1.7 times lower IC_{50} compared to Vitamin C. In conclusion, antioxidant activity of BMC was found to be superior to Curcumin and Vitamin C in all tested antioxidant assays.

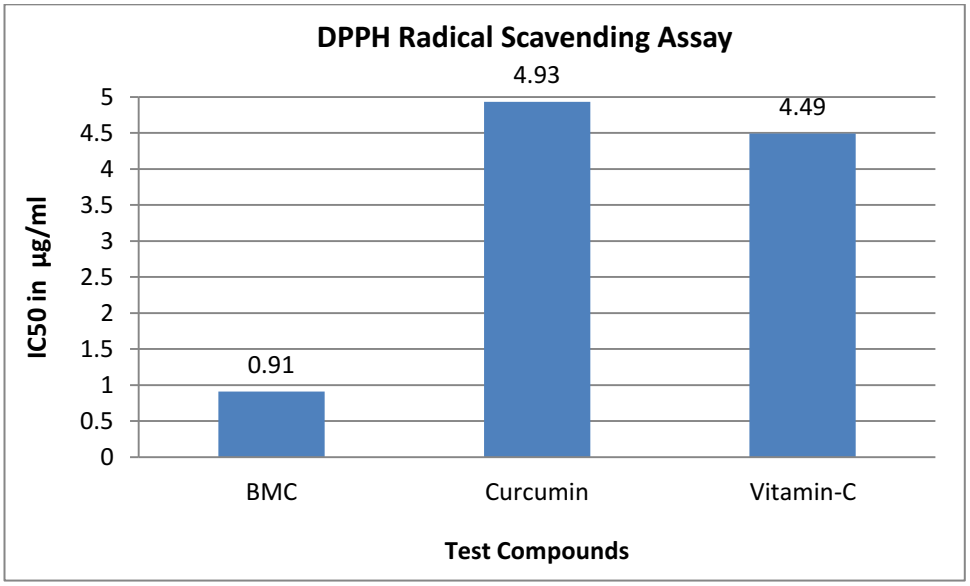

Figure 16. Results from DPPH radical scavenging activity assay

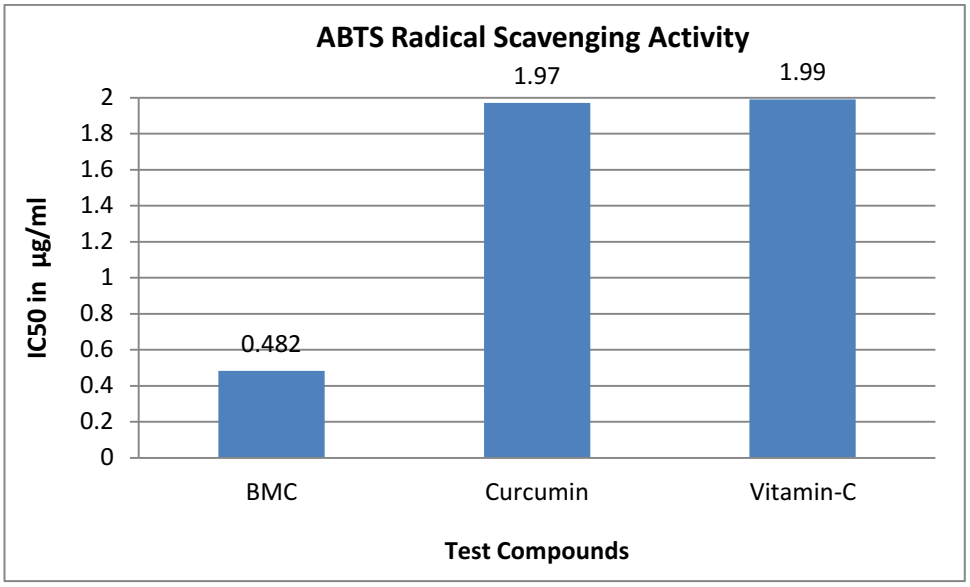

Figure 17. Results from ABTS radical scavenging activity assay

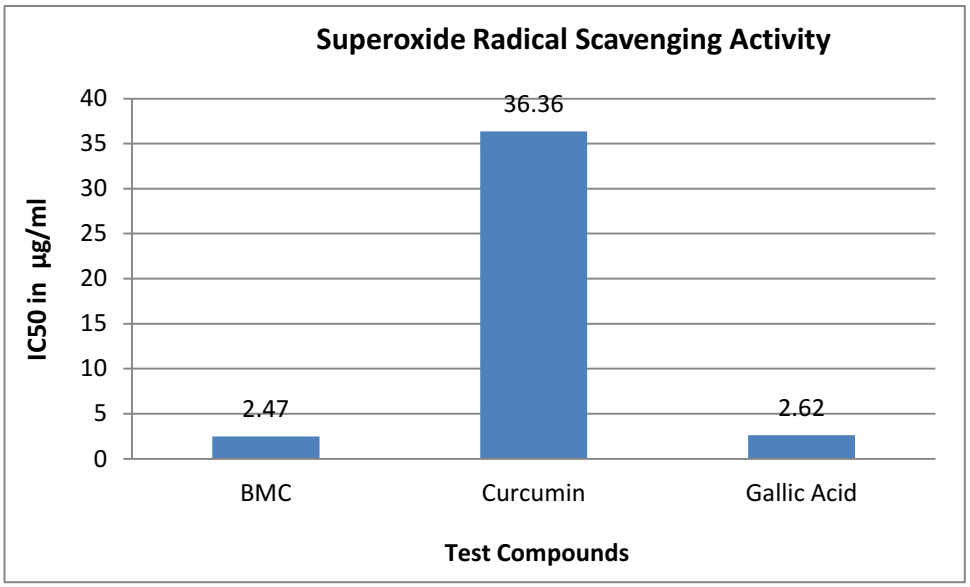

Figure 18. Results from Superoxide radical scavenging activity assay.

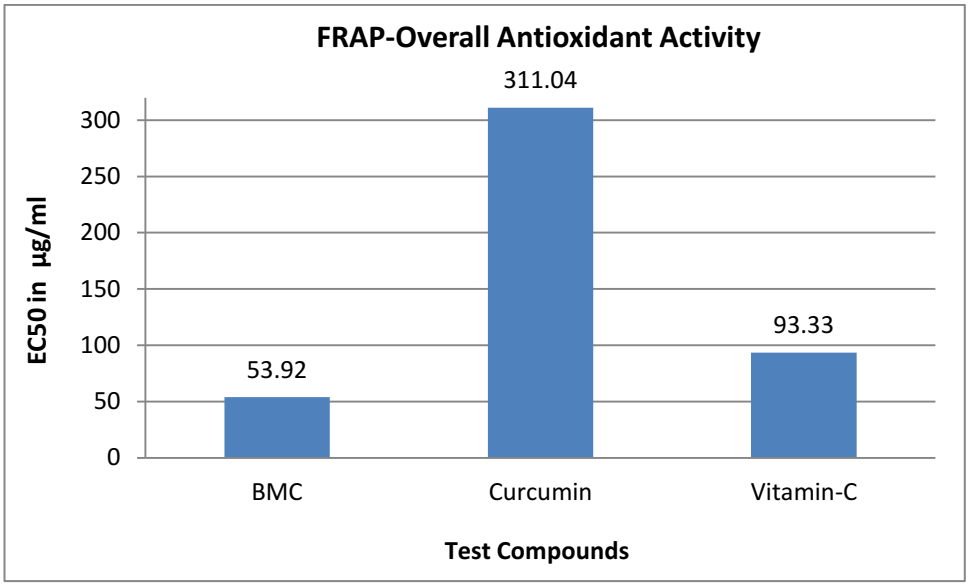

Figure 19. Results from FRAP Overall antioxidant activity assay

Table 16. Number of time increase in antioxidant activity for BMC in comparison with Reference compound

Name of the Assay	Reference Compound	No. of time increase in antioxidant activity for BMC in comparison with Reference compound
ABTS Assay	Curcumin	4.1
	Vitamin C	4.1
DPPH Assay	Curcumin	5.4
	Vitamin C	4.9
Super oxide radical scavenging Assay	Curcumin	14.7
	Gallic acid	1.1
FRAP Assay	Curcumin	5.8
	Vitamin C	1.7

5.4.2 Evaluation of anti-inflammatory properties:

a. 5-Lipoxygenase inhibition by BMC

EC_{50} values for BMC and Curcumin in the inhibition of 5-Lipoxygenase (LOX) were found to be 6.58 µg/ml and 27.47 µg/ml respectively and are shown in Fig. 20. BMC was found to be 4.5 times more potent inhibitor of 5-Lipoxygenase than curcumin.

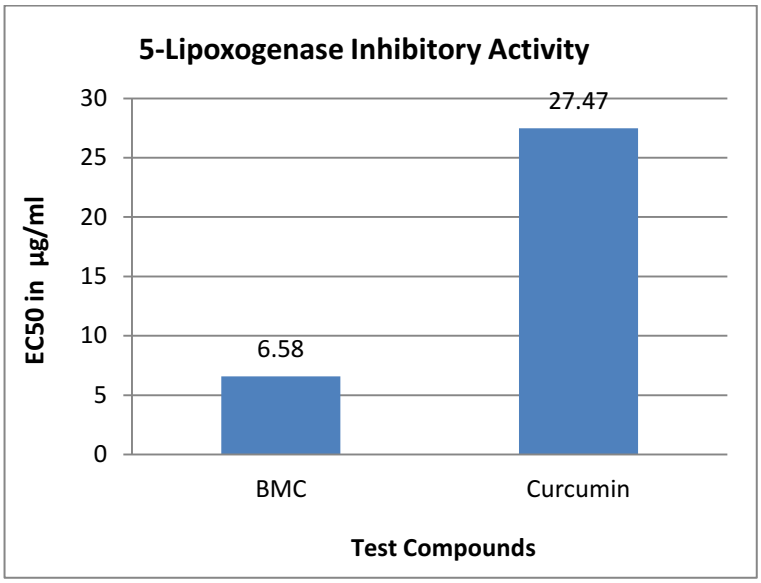

Figure 20: 5-Lipoxygenase inhibitory activity of BMC and Curcumin

b. Evaluating inhibition of cytokine (TNF-α, IL-1β and IL-6) production by BMC in lipopolysaccharide (LPS) stimulated whole Blood.

IC_{50} values of Curcumin were found to be 90.93 µg/ml, 137.4 µg/ml and 32.96 µg/ml for TNF-α, IL-6 and IL-1β respectively, where as for BMC, it was found to be 88.27 µg/ml, 119.9 µg/ml and 26.94 µg/ml for TNF-α, IL-6 and IL-1β respectively and the results shown in Table 17. BMC has shown more potent anti-inflammatory activity when compared to Curcumin in *in vitro* studies.

Table 17. IC_{50} values of BMC and Curcumin for pro-inflammatory cytokines in LPS induced whole blood.

Name of the test Compound	IC_{50} - µg/ml		
	TNF-α	**IL-6**	**IL-1β**
BMC	88.27	119.9	26.94
Curcumin	90.93	137.4	32.96

5.4.3 Beta-Secretase (BACE1) inhibition by BMC:

Only BMC was evaluated for the inhibitory activity against BACE1 as the Curcumin was already being tested by others. BACE1 inhibitory effect of BMC was evaluated using PanVera®'s BACE1 fluorescence resonance energy transfer (FRET) Assay Kit (2985). IC_{50} value was estimated to be 1.471 µg/ml for BMC. Lower IC_{50} value indicates that BMC is one of the potent inhibitor of BACE1 and hence can be a potential candidate for treatment of Alzheimer's disease.

5.5. Summary:

In *in vitro* studies, BMC was found to have superior antioxidant activity compared to its parent compound Curcumin and reference compound Vitamin C and Gallic acid. It was shown that BMC is a potent antioxidant and anti-inflammatory agent and has several folds better antioxidant and anti-inflammatory activity compared to Curcumin. Additionally, BMC has exhibited very good BACE1 (validated drug target of Alzheimer's disease) inhibitory activity (IC_{50}=1.471 µg/ml) which makes it more unique in terms of multipotent drug candidate compared to other antioxidant and anti-inflammatory molecules. These *in vitro* findings have to be further tested in appropriate animal model study to prove the efficacy of BMC for application in Alzheimer's disease.

Chapter-6

6. Formulation development for BMC:

6.1 Introduction:

Formulation development is one of the crucial steps in the successful drug development process. Many of the drug candidates are found to have poor aqueous solubility and hence poor oral bioavailability, leading to the drug failure in *in vivo* studies. Similar to this, one of the major problem associated with the use of phytochemicals as drug candidate is their poor bioavailability and hence, poor therapeutic efficiency in *in vivo.* Curcumin is one such phytochemical known to have poor aqueous solubility and poor oral bioavailability. Like parent compound, BMC was also found to be insoluble in water and hence predicted to have poor bioavailability. Therefore, there is a need for development of suitable formulation for BMC which can improve its aqueous solubility and hence, bioavailability.

According to the Biopharmaceutical Classification System (BCS), hydrophobic drugs fall into two groups depending on their solubility and permeability across cell membranes i.e. Class IV (poor solubility but high permeability) or Class V (poor solubility and low permeability) (The Biopharmaceutics Classification System (BCS) Guidance., http://www.fda.gov). The drugs with poor solubility and low permeability exhibit poor pharmacokinetics thereby leading to poor efficacy. These drugs would show improved pharmacokinetics if their aqueous solubility is improved. About 70% of the new drug candidates showed poor solubility limiting their *in vivo* bioavailability (Khadka *et al.,* 2014). Solubility is considered as rate limiting factor in oral bioavailability (Savjani, 2012). The most common method of enhancing the solubility is by converting them into their salts or adding an ionisable centre like an amine group (Waterbeemd *et al.,* 2009). But in these cases, extensive toxicity studies need to be done in order to establish that the modified molecule does not contain any untoward side-effects.

The use of surfactants to solubilize hydrophobic drugs has been widely reported. The selection of surfactants is an important criterion as surfactants lower the interfacial tension between water and the hydrophobic drug thereby enhancing the drug solubility in water (Savjani, 2012). Surfactants form supramolecular aggregates called micelles after a particular concentration in water and is called the critical micelle concentration (CMC). The solubilization mainly takes place through the formation of micelles and hence it is called micellar solubilization. Surfactants with a hydrophilic-lipophilic balance (HLB) value of >15 are appropriate for the purpose as they form stable oil in water emulsions. It is also a well known fact that large amounts of ionic surfactants cause irritation and hence the focus is now on nonionic surfactants as they do not cause irritation and also have low CMC than ionic surfactants (Lawrence *et al.,* 2000). Use of combinations of surfactants and co-surfactants enhances the solubility to a larger extent (Liu and Guo, 2007). It has been reported that the increase in solubility may be attributed to the lowering of interfacial tension, greater penetration of the hydrophobic phases into the surfactant monomers and an increase in the fluidity of the interface between the hydrophobic phase and water (Warisnoicharoen *et al.,* 2000; Lawrence *et al.,* 2000). But the main drawback of this method was that larger amounts of surfactants would be needed to solubilize drug molecule (Liu and Guo, 2007).

There are several techniques explored for improving the aqueous solubility and oral bioavailability of poorly bioavailable drugs such as micronization, nanosuspension, emulsions, cocrystallization, solid dispersion, complexation, derivatization and use of surfactants (Savjani, 2012). Among them, nanotechnology based formulations are popular due to their higher rate of success. Self emulsifying drug delivery system (SEDDS) has been used for increasing the solubility and bioavailability of drug with great success. SEDDS are isotropic mixture of lipids, surfactants and co-solvents, which forms oil in water emulsion in aqueous media, with droplet size ranging from nanometers to micrometers (Sarpal *et al.,* 2010; Pujara, 2012; Nigade *et al.,* 2012; Rigon *et al.,* 2015). Nonionic surfactants such as polysorbates are preference for SEDDS due to their lower CMC. SEDDS are more suitable for the drugs with cLogP value between 2 and 4 (Sarpal *et al.,* 2010). The cLogP value for BMC was found to be 2.44 during the *in silico* studies and was in the range specified by Sarpal *et al., 2010* for SEDDS. Thus the objective of current study was to develop a Self emulsifying drug delivery system (SEDDS) for BMC to enhance its aqueous solubility. Polysorbate 80

and PEG 400 were used as surfactant and co-surfactant, respectively for the formulation development. Developed formulation had good solubility for BMC in water. Further, developed formulation of BMC was characterized using particle size and Transmission Electron Microscope (TEM) analysis.

6.2. Research work:

The present chapter work aims at developing a Self emulsifying drug delivery system (SEDDS) for BMC to enhance its aqueous solubility. Further, developed formulation of BMC was characterized using particle size and Transmission Electron Microscope (TEM) analysis.

6.3. Materials and Methods adopted:

6.3.1 Materials:

BMC (95%) was obtained from Laila Pharmaceuticals Pvt. Ltd., Tween 80 (Merck), PEG 400 (Loba), were used for formulation development. MilliQ water, methanol (HPLC grade), acetonitrile (HPLC grade) , Orthophosphoric acid (HPLC grade) were obtained from Merck; (Merck).

The materials used for cellular update study are; BMC formulation, Minimum Essential Media with 20% Fetal Bovine Serum (MEM with 20% FBS), 0.25% Trypsin/EDTA, Phosphate Buffer Saline (pH 7.4), Methanol, BCA (Bicinchoninic Acid) reagent, and Caco-2 cell line (Sigma Aldrich Catalogue No. 86010202 Lot No. 10J006).

6.3.2 Methods:

6.3.2.1 Preparation of BMC formulation:

SEDDS technology was selected for BMC formulation development. SEDDS are more suitable for the drugs with LogP value between 2 and 4 (Sarpal *et al.,* 2010). The LogP value for BMC was found to be 2.44, which is in the range specified by Sarpal *et al* for SEDDS. Ingredients were selected based on literature review and HLB value for surfactants (Anjana *et al.,* 2012). Nonionic surfactants such as polysorbates are preference for SEDDS due to their lower CMC; accordingly polysorbate 80 (HLB=15)

was selected as surfactant and PEG 400 as cosurfactant. Compatibility of surfactants and co-surfactants was tested by mixing them together in different combinations and concentration and observed for phase separation after centrifuging at 3000 rpm for 10 minutes. After arriving at right combination of surfactant and cosurfactant, BMC was dissolved in co-surfactant and surfactant mixture with the help of ultrasonication (20 KHz, 20 minutes). Different concentration of BMC ranging from 1 to 10% was evaluated for solubility using sonicator.

6.3.2.2 Analysis of BMC percentage in formulation by High Performance Liquid Chromatography (HPLC):

BMC analysis was carried out as per the instructions given by the supplier. The HPLC (Shimadzu, Japan) with C-18 column (250mm length, 4.6mm diameter) was used for the estimation, and analysis of BMC in the formulation. The HPLC conditions used were as follows; Mobile phase 52 (0.1% phosphoric acid): 48 (Acetonitrile), Flow rate of 1ml/minute and detector was set at 424nm. Twenty µl of sample was injected for every run. The preparations of samples were done briefly as below: Accurately weighed 30 mg of BMC formulation was taken and made up to 50 ml using methanol. Different concentrations of standard and samples were prepared. All samples were filtered through 0.2µ filters (Rankem). Standard protocol was followed for the determination of BMC concentration in the formulation.

6.3.2.3. Characterization of BMC formulation –Size, Morphology:

The prepared nanoemulsion was characterized using a particle size analyzer and Transmission Electron Micrography (TEM).

a. Particle size Analysis

The BMC formulation particle size in water was measured by Dynamic light scattering (DLS) using Malvern particle size analyzer. Five mg of BMC formulation were dissolved in 1,000 ml of water, 3ml of this solution was filled in a syringe fitted with a 0.2µm filter and filtered into a cuvette. Cuvette was placed into the reading chamber of the analyzer and sizes of the particles were recorded.

b. Transmission electron microscopy (TEM)

The morphology of the BMC formulation in water was studied using TEM. Ten mg of BMC formulation was mixed with water (1000 ml) to get a clear solution. One drop of BMC solution was added onto copper grid and was allowed to dry and then was read under TEM at 200,000 X magnifications (JEOL1200EX TEM) and photographs were taken.

6.3.2.4 Cellular update of BMC by Caco2 cells:

Cellular update of BMC from its formulation was studied in Caucasian colon adenocarcinoma (Caco2) cells (Kunwar *et al.,* 2006; Kunwar *et al.,* 2007). The procedure followed is;

1. Caco-2 cells were grown in 75 cm^2 flask at 37ºC/ 5% CO_2
2. At 85-90 % confluence, cells were trypsinized and centrifuged at 2500 rpm for 5min.
3. Cells were suspended in 10 ml of MEM containing 20% FBS and counted
4. 1 million cells were seeded in each of the 35mm cell culture plates and allowed to attach and grow overnight at 37ºC/ 5% CO_2.
5. At 75-80% confluence, plates were randomly assigned as treatment groups and control. The treatment group cells were treated at 80, 50 and 25 µM BMC formulation. Control groups cells were treated with vehicle of the BMC formulation.
6. Treatment was carried out for one hour, subsequently cells were separated and pelleted by centrifugation.
7. Cells were washed twice in PBS and reconstituted in 100 µl of PBS.
8. From the above cell suspension 10 µl of cell suspension was taken for protein estimation. The protein estimation was done as follows.
    a. 10µl of Lysis buffer (PBS containing 2% TritonX100) was added to 10µl cell suspension
    b. Sonicated in sonication bath for 10 min and subsequently centrifuged and supernatant was collected.
    c. Protein content was estimated using BCA reagent.
10. The remaining 90 µl cell suspension was processed as follows

a. Cells were centrifuged and pellet was collected
b. The pellet was allowed to dry for 10 mins.
c. The pellet was suspended in 250 µl of methanol and sonicated in a sonication bath for 10 mins.
d. After centrifugation the supernatant was collected and analyzed for BMC by HPLC
e. Blank readings were subtracted from all data and each reading was normalized by individual protein concentration. Final results were expressed as mean±SD of µM BMC uptake /mg protein

6.4. Results:

6.4.1 Formulation development:

BMC has poor aqueous solubility and hence it was essential to develop formulation to enhance its aqueous solubility. SEDDS are more suitable for the drugs with cLogP value between 2 and 4 (Sarpal *et al.,* 2010). The LogP value for BMC was found to be 2.44 during the *in silico* studies and was in the range specified by Sarpal *et al* for SEDDS and hence, SEDDS was selected for formulation development. Nonionic surfactants such as polysorbates are preference for SEDDS due to their lower CMC, accordingly polysorbate 80 (HLB=15) was selected as surfactant and PEG 400 as cosurfactant. The surfactant was selected based on literature evidence, safety and their HLB (Hydrophile-Lipohile Balance) value which governs the BMC solubility and its ability to form appropriate O/W nanoemulsion in water. Compatibility and solubility of BMC was tested in various ratio of 1:1. 2:1, 3:1, 4:1 and 5:1. As the concentration of Polysorbate increased, solubility of BMC also increased up to the ratio of 4:1 of polysorbate and PEG 400. Thereafter there was no increase in amount of BMC solubility as per visual observation for BMC residue. Polysorbate 80 and PEG 400 were selected for BMC formulation as surfactant and cosurfactant, respectively in 4:1 ratio. After arriving at right a combination of surfactant and cosurfactant, BMC was dissolved in co-surfactant and surfactant mixture with the help of ultrasonication (20 KHz, 20 minutes) as shown in the Figure 21. The final composition of BMC formulation is given in Table 18. BMC formulation was found to be viscous dark red colored solution (Fig 22) containing 5.67% BMC (Table 19; Fig 24). It was found to

have good solubility in water and formed clear yellow colored emulsion in water at the concentration of 1mg/ml (Fig 23). This formulation was used for further studies including animal model study.

Table 18: Composition of BMC formulation

Sl No	Ingredients	Amount (g)/100 g
1	BMC	6
2	Polysorbate 80	75.2
3	PEG 400	18.8

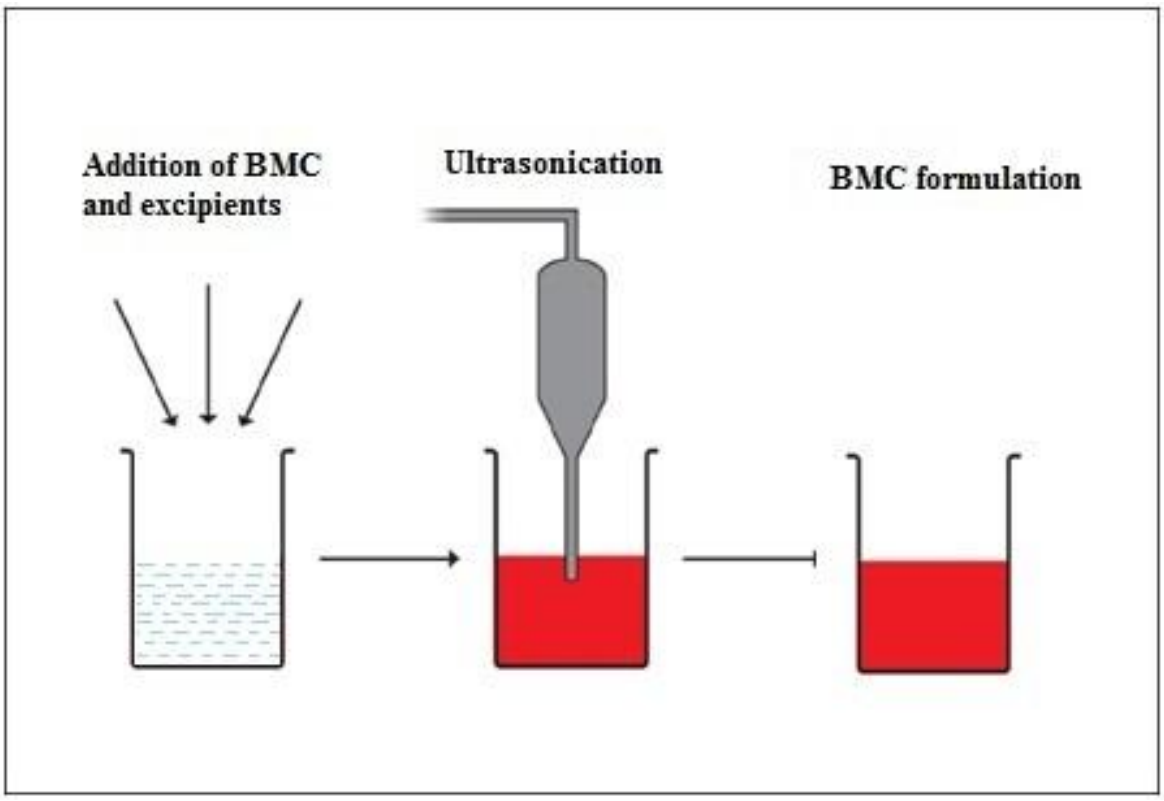

Figure 21. Schematic diagram of manufacturing process for BMC formulation.

Figure 22. Photograph of BMC formulation.

Figure 23. Photograph of BMC formulation in water showing complete solubilization of BMC.

Table 19: Physical and chemical properties of BMC formulation.

Sl No	Parameters	Test results
1	Appearance	Clear liquid
2	Color	Deep red
3	BMC concentration (%)	5.67

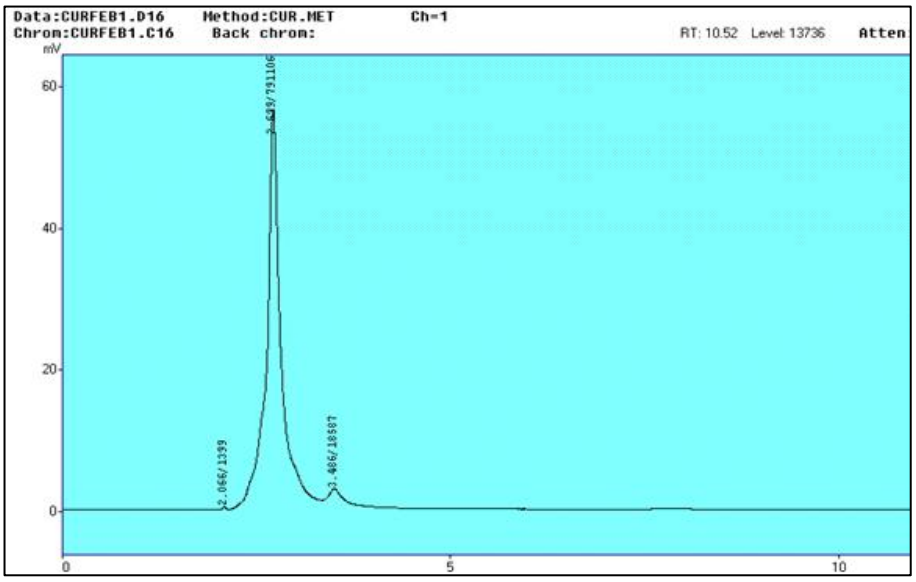

Figure 24. HPLC chromatograph for BMC

6.4.2 Characterization of BMC formulation -Size and Morphology:

6.4.2.1 Particle size analysis:

The particle size of the BMC formulation in water was determined by Malvern particle size analyzer. The mean diameter of the particle was found to be 254 nm.

6.4.2.2 Transmission Electron Microscopic (TEM) analysis:

TEM analysis of BMC formulation in water showed that, the formulation is forming microemulsion in water and the particles are in spherical shape (Fig 25).

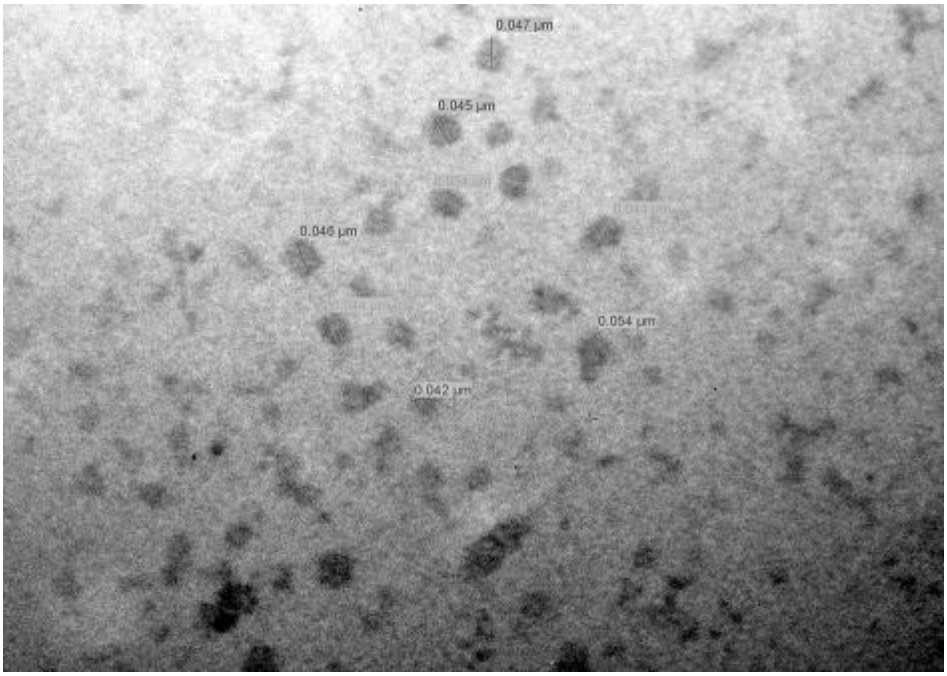

Figure 25. TEM image of BMC formulation in water

6.4.3 Cellular update of BMC by Caco2 cells:

BMC has shown good uptake in Caco2 cells lines and the results are shown in Figure 26.

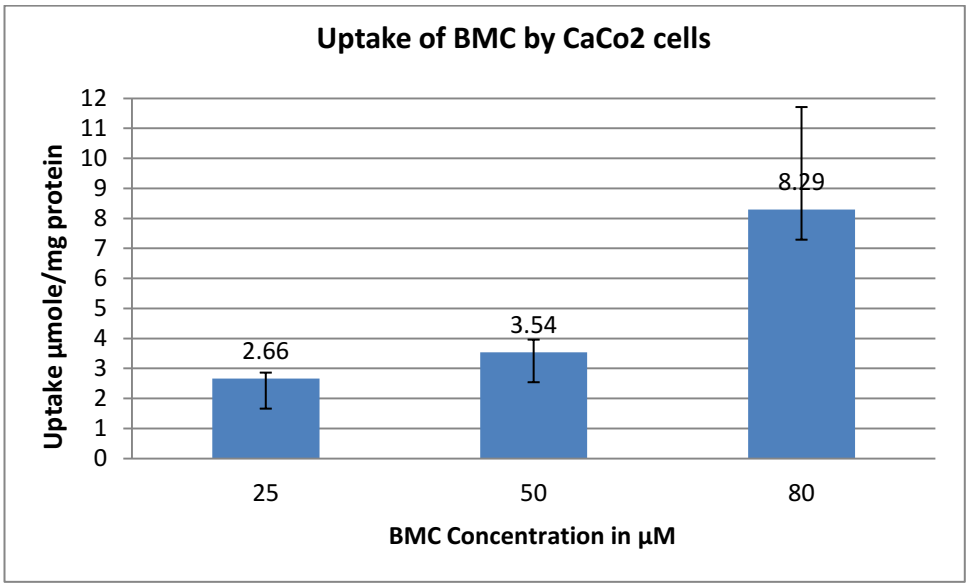

Figure 26: Cellular uptake of Formulated BMC in Caco-2 cells.

6.5 Summary:

Self emulsifying drug delivery system was developed for BMC using polysorbate 80 and PEG 400 at the ratio of 4:1 using ultrasonication. The final concentration of BMC in the formulation was found to be 5.67%. Ultrasonic solubilization occurs through acoustic cavitation of the Smix (Li *et al.,* 1978). When this formulation was added into water (1mg/ml of water), it underwent self emulsification to form microemulsion containing BMC. It was completely soluble at the concentration level of 1 mg /ml in water forming microemulsion with the particle size of 254 nm. It was reported that the self emulsifying drug delivery systems forms microemulsion in water with the particle size ranging from 100 to 250 nm (Sarpal *et al.,* 2010). The average particle size of BMC formulation in water was found to be around 254 nm, which is slightly higher than the reported particle size for SEDDS formulations. The particles are found to be spherical in shape as shown in transmission electron micrograph pictures.

Chapter-7

7. Evaluation of efficacy of BMC in *in vivo* animal model study:

7.1 Introduction:

A successful and cost effective therapeutic for Alzheimer's disease can be achieved only through combining *in silico*, *in vitro* and *in vivo* analyses (Pelkonen *et al*., 2011). *In silico* approach can help to reduce the cost of conventional screening, subsequently *in vitro* studies can be used for validating *in silico* results, finally *in vivo* experiments reveals the feasibility of a potential human application. There were many attempts made to model *in vivo* physiopathological events of Alzheimer's disease in animals (Langley, 2014). There are many animal models used in the research of Alzheimer's disease. These animal models include primates, dogs, ageing rats and chemical induced rodents, newer models include genetically modified zebrafish and *Caenorhabditis elegans* (Newman *et al.,* 2007; Langley, 2014). Dog is considered as one of the best model for studying ageing human brain and neurological disease however, their use is limited by availability, cost and ethical reasons. Chemically induced animal models such as scopolamine induced amnesia model mimicking the specific pathophysiological process of Alzheimer's disease are explored for screening the drugs. These models are based on cholinergic hypothesis of Alzheimer's disease, which is not involved in development of Alzheimer's disease. Aβ induced rodent models based on amyloid cascade hypothesis are developed and explored for the drug development but they are considered as partial model of Alzheimer's disease (Dam and Deyn, 2011). Several genetically modified species such as mice, rats, zebrafish, nematodes and fruits flies are used in the past. The majority of experiments were conducted using transgenic mice (Tg mice) (Gotz and Ittner, 2008; Dam and Deyn, 2011; Langley, 2014). Most of the Tg mice carry one or more human genes related to amyloid hypothesis of Alzheimer's disease (Howlett, 2011; Shineman *et al.,* 2011).

Apart from the above mentioned animal models, aluminium chloride-induced Alzheimer's disease rat models are also used in the preclinical studies of Alzheimer's disease drugs. Aluminium chloride is a neurotoxin which induces many Alzheimer's disease pathology related physiological changes in the brain (Boegman & Bates, 1984; Nehru & Anand, 2005). Aluminium chloride-induced neurotoxic effect is mainly due to oxidative damage caused by elevated levels of free radicals (Boegman & Bates, 1984; Donald, 1989; Lebel & Bondy, 1991; Yokel, 2001). Elevated free radicals are known to induce formation of NFTs, Aβ plaques and lipid peroxidation in the brain (Nehru & Anand, 2005). Lipid peroxidation is one of the major causes of many neurological disorders including Alzheimer's disease. Other Alzheimer's disease like features induced by aluminium chloride are, increased levels of Aβ protein, degeneration of cholinergic terminals in cortex and hippocampus, and finally neuronal apoptosis (Khan *et al*., 2013). Also, the accumulation of aluminium in hippocampal region impairs hippocampal synaptic plasticity leading impaired cognitive functions (Lal *et al*., 1993; Julka *et al*., 1996). In this current research work, aluminium chloride-induced rat model of Alzheimer's disease was used for the evaluation of *in vivo* neuroprotective efficacy of BMC.

7.2 Research Work:

The present chapter work was aimed at evaluating efficacy of BMC using aluminium chloride-induced rat model. Briefly, Alzheimer's disease symptoms were induced in Sprague-Dawley rats using administration of aluminium chloride. In this animal model, various parameters have been investigated to understand the disease progression which includes, estimation of lipid peroxidation and superoxide dismutase activity in the brain, circulating superoxide dismutase activity in blood and RBC lysate, protein phosphatase 2A (PP2A) activity as well as histopathology of brain. The results of lipid peroxidation and superoxide dismutase activity indicate the oxidative stress induced by aluminum chloride via generation of free radicals. PP2A activity is another important parameter that can be correlated to the Alzheimer's disease progression. Tau hyperphosphorylation in Alzheimer's disease is thought to be caused by a decrease in PP2A activity. Hence, determining the PP2A activity in the brain sample also clarifies the extent of disease progression. Finally, histopathology of brain was performed in order to locate the probable sites of degeneration. These analyses using the above mentioned aluminium

chloride- induced rat model will provide preliminary understanding of the efficacy of BMC and its safety profile. In conclusion, the present chapter describes the methodology, results observed and appropriate inferences from these *in vivo* investigations.

7.3 Materials and Methods adopted:

7.3.1 Animal Model:

In this current research work, aluminium chloride-induced rat model was used to evaluate the neuroprotective efficacy of BMC. Alzheimer's disease was induced in Sprauge-Dawley rats using aluminium chloride (Dose: 4.2 mg/kg bodyweight/day, intraperitonially daily for 28 days) (Nikvsarkar *et al.,* 2006). In this animal model, various parameters have been evaluated to understand the disease progression such as, estimation of lipid peroxidation in brain, superoxide dismutase activity in brain, circulating superoxide dismutase activity in blood, protein phosphatase 2A (PP2A) activity and histopathology of brain. This model is suitable for preliminary evaluation of efficacy of anti-Alzheimer's disease drug.

7.3.2 Materials:

Compound : BMC (Formulation containing BMC)

Vehicle : Solution containing Polysorbate 80 and PEG 400

7.3.3 Animals:

Sprague-Dawley rats male (n=18) weighing 166.50±3.70 g and female (n=18) weighing 162.33±2.49 g were used for the study. The animals were housed in controlled environment having relative humidity of 60 % and temperature of 20°C. Animals were fed with 40g of rodent diet per animal per day and tap water provided. Laboratory used for the animal model study is registered with CPCSEA, Ministry of Social Justice and Empowerment, Government of India and proper ethical committee approval was obtained before the initiation of the study.

7.3.4 Ethics committee approval:

Institutional ethical committee approval (from B.V. Patel PERD centre, India) was obtained before the initiation of the study and the approval number was TOX200411.

7.3.5 Study Design:

a. Acclimatization:

The animals were kept in housing cages for one week and allowed to acclimatize in the laboratory before conducting the study. The health status were monitored before the start of the study (Nikvsarkar *et al.,* 2006).

b. Randomization:

Total randomization was done for each sex, random cage numbers were split into sequential blocks and one block was assigned to each dose level.

c. Dosage groups and dose levels:

Three groups of animals (n=12) of both the sexes were divided into following treatments;

Table 20. Animal groups and dosage level

Sl no	Animal Group	Dosage
1	Control group	Vehicle 8.87g/kg body weight (In two divided doses)
2	Disease group	$AlCl_3$ 4.2 mg/kg body weight (Once in a day)
3	Test Group	$AlCl_3$ 4.2 mg/kg body weight (Once in a day) and BMC 500mg/kg body weight (BMC equivalent) (In two divided doses)

d. Administration of drug/vehicle:

The animals were administered the test compound/vehicle as an oral dose every day in the morning and evening hours for 28 days. Aluminium chloride was dissolved in normal saline and then injected through intra-peritoneal route in rats (Nikvsarkar *et al.,* 2006).

e. **Study schedule:**

Groups of 6 male and 6 female rats were administered BMC by oral dosage for a period of 28 days in parallel to $AlCl_3$ by intra-peritoneal route (Table 21).

Table 21**.** Animal study design

Treatment Groups	Dosage	Male rats	Female rats
Control Group	Vehicle 8.87g/kg (In two divided doses)	6	6
Disease Group	$AlCl_3$ 4.2 mg/k g (Once in a day)	6	6
Test (BMC) Group	$AlCl_3$ 4.2 mg/kg (Once in a day) and BMC: 500mg/kg - API equivalent ;In two divided doses	6	6

f. **Dose Formulation:**

Formulation of BMC (As detailed in Chapter 6), containing BMC as active pharmaceuticals ingredient was used in this study.

7.3.6 Observations : The following observations (Table 22) were carried out during the study

Table 22.Observation parameters in the animal study

Observation Parameters	Day of study	Treatment Groups
Body Weight	Before start of treatment, daily and end of treatment	All
Clinical Signs	Day 1 every 2 hr. up to 8 hrs and daily twice a day till the end of treatment	All

Mortality/Moribund	Daily	All
Haematology	End of treatment	All
Necropsy & Organ Weight	End of treatment	All
Histopathology	End of treatment	All

7.3.6 Assessment procedure:

7.3.6.1 Clinical signs and mortality:

The external appearance and behavior of the animals were recorded daily. The animals showing severe signs of intoxication were sacrificed and subjected to necropsy and anatomopathological examination, as well as, in any animal found dead (Lalla and Shah, 2010).

7.3.6.2 Body weight and food consumption:

All the animals were weighed daily during the study. The dosage was calculated everyday according to the weight of the animal on that particular day. Forty gram food was provided per animal daily.

7.3.6.3 Blood and serum biochemistry analysis:

At the end of the treatment period, the animals were fasted overnight and blood samples were collected by retro orbital sinus bleeding into heparinized (for haematology) and non-heparinized tubes (for serum collection, for clinical biochemistry) (Lalla and Shah, 2010) and the following parameters were assessed:

Blood: RBC, WBC, Haematocrit, MCV, MCH, MCHC, Haemoglobin, Platelets and differential leukocyte count. Haematology was performed using a Sysmax KX 21 automated haematology analyzer.

Serum: Glucose, Glutamyl Pyruvate Transaminase (SGPT), Glutamyl Oxaloacetate Transaminase (SGOT), Creatinine, Urea, Cholesterol, Triglyceride, Total Protein and Albumin.

The serum biochemistry was performed using diagnostic kits supplied by Transasia auto kits on an EM 360 automated clinical biochemistry analyzer.

7.3.6.4 Lipid peroxidation (LPO):

Lipid peroxidation in the brain was determined following the method reported by Ohkawa *et al*., 1979 and Nikvsarkar *et al.,* 2006 using thiobarbituric acid and measuring the absorbance at 532 nm. The lipid peroxidation value was expressed in nmol malonaldehyde per gram of fresh brain tissue.

7.3.6.5 Superoxide dismutase (SOD):

SOD activity was determined in brain, plasma and RBC lysate following the method reported by Marklund and Marklund, 1974, and Nikvsarkar *et al.,* 2006. RBC lysis was carried out by taking 1 ml of heparinized blood and centrifuged at 2500 rpm for 30 min at 4°C. The plasma was then carefully separated. The erythrocyte pellet was washed with normal saline for three times using centrifugation, then were diluted with water (4 ml) to lyse the erythrocytes. Haemoglobin was separated by adding 1 ml of ethanol and 0.6 ml of chloroform. The above mixture was mixed for 15 min and centrifuged at 2,500 rpm for 10 minutes at cold condition (4^{o}C). Water ethanol layer was aspirated and diluted with 0.7 ml of double distilled water. Superoxide dismutase activity was measured in the aqueous-alcohol layer (100 µl) using pyrolol as substrate.

7.3.6.6 PP2A activity:

PP2A activity in the brain of animal was performed using serine-threonine phosphatase assay kit (Promega, Catalogue No. V2460). The procedure described in the user's manual was followed for the assay. The total protein was determined using micro-BCA protein assay kit (As per Thermo scientific, Catalogue No. PI23235).

7.3.6.7 Pathological Anatomy:

All the animals in the study were subjected to a full autopsy and the following organs were weighed: Brain, Lungs, Liver, Heart, Kidney, Spleen, Adrenals, Testis and Epididymis (in males), Ovary and Uterus (in females). Brain was preserved for histopathological examination in all groups.

7.3.6.8 Histological Procedure:

The method followed involved the fixation in 10% formalin, 5 μm sectioning, staining with haematoxylin and eosin and microscopic examination for all possible changes in the anatomy (Shidham *et al.,* 2001).

7.3.6.9 Statistical Analysis:

Statistical analysis was done applying one-way ANOVA followed by Bonferroni Multiple Comparisons Test. P value ≤ 0.05 is considered to be significantly different. All the values are expressed as Mean±SE.

7.4 Results:

All the results are expressed as Mean±SE. All the animals were survived in all the groups during the study and the results are shown in Table 23A and 23B. No clinical signs of intoxication were noticed during the study in animals of all the groups and the results are shown in Table 24A and 24B. The clinical signs included appearance, respiration, motor activity, prostrations, tremors and convulsions, reflexes (corneal, primordial), ocular changes (lacrimation, ptosis, mydriasis/miosis), salivation and diarrhea.

Body weights of all the animals were recorded as per the study design and the weekly average body weights per group are shown in Table 25A, Table 25B, Figure 27 and Figure 28. Average weekly body weight of the animal showed a gradual increase in body weight in the animals of all three groups. In test group there was a loss of body weight on 21st day, and then it increased at the end of the study.

Absolute organ weight of all animals that has undergone necropsy showed similar pattern in all the groups and the results are shown in Table 26A and 26B. No significant change was seen in any of the groups.

Summary of hematological data is given in Table 27A and 27B. All the hematological data obtained from animals of all groups were within the normal range.

Summary of clinical biochemistry data is given in Table 28A and 28B. All the clinical biochemistry data obtained from animals of all groups were within the normal range.

Summary of gross necropsy data is given in Table 29A and 29B.Gross necropsy did not show any abnormality in all the animals belonging to all three groups.

Summary of histopathological data is given in Table 30A, 30B and 30C. Brain sections in all the animals of all the groups showed histology of brain tissue with normal nerve cells and glial tissues.

Histological picture of brain samples of animals from all the groups are shown in Figure 29 to Figure 34.

Summary of brain LPO data of all three groups is given in Table 31A, Table 31B, Table 31C, Figure 35 A and Figure 35 B. Summary of SOD data in RBC lysate is given in Table 32A, Table 32B, Table 32C, Figure 36A and Figure 36B. Summary of circulating SOD data in blood plasma is given in Table 33A, Table 33B, Table 33B, Figure 37A and Figure 37B. Summary of brain SOD data is given in Table 34A, Table 34B, Table 34C, Figure 38A and Figure 38B. Summary of PP2A enzyme activity data is given in Table 35A, Table 35B, Table 35C, Figure 39A and Figure 39A.

Table 23A: Animal survival data- Male rats

Group	**Control group**	**Disease group**	**Test group**
Experimental days	**No. of surviving rat / initial number of rats**		
1	6/6	6/6	6/6
2	6/6	6/6	6/6
3	6/6	6/6	6/6
4	6/6	6/6	6/6
5	6/6	6/6	6/6
6	6/6	6/6	6/6
7	6/6	6/6	6/6
8	6/6	6/6	6/6
9	6/6	6/6	6/6
10	6/6	6/6	6/6
11	6/6	6/6	6/6
12	6/6	6/6	6/6
13	6/6	6/6	6/6
14	6/6	6/6	6/6
15	6/6	6/6	6/6
16	6/6	6/6	6/6
17	6/6	6/6	6/6
18	6/6	6/6	6/6
19	6/6	6/6	6/6
20	6/6	6/6	6/6
21	6/6	6/6	6/6
22	6/6	6/6	6/6
23	6/6	6/6	6/6
24	6/6	6/6	6/6
25	6/6	6/6	6/6
26	6/6	6/6	6/6
27	6/6	6/6	6/6
28	6/6	6/6	6/6

Table 23B: Animal survival data- Female rats

Group	Control group	Disease group	Test group
Experimental days	**No. of surviving rat / initial number of rats**		
1	6/6	6/6	6/6
2	6/6	6/6	6/6
3	6/6	6/6	6/6
4	6/6	6/6	6/6
5	6/6	6/6	6/6
6	6/6	6/6	6/6
7	6/6	6/6	6/6
8	6/6	6/6	6/6
9	6/6	6/6	6/6
10	6/6	6/6	6/6
11	6/6	6/6	6/6
12	6/6	6/6	6/6
13	6/6	6/6	6/6
14	6/6	6/6	6/6
15	6/6	6/6	6/6
16	6/6	6/6	6/6
17	6/6	6/6	6/6
18	6/6	6/6	6/6
19	6/6	6/6	6/6
20	6/6	6/6	6/6
21	6/6	6/6	6/6
22	6/6	6/6	6/6
23	6/6	6/6	6/6
24	6/6	6/6	6/6
25	6/6	6/6	6/6
26	6/6	6/6	6/6
27	6/6	6/6	6/6
28	6/6	6/6	6/6

Table 24A : Summary of clinical signs - Male rats

Sl. No	Treatment group	Clinical signs
1	Control Group (n=6)	No Abnormality detected
2	Disease Group (n=6)	No Abnormality detected
3	Test Group (n=6)	No Abnormality detected

Table 24B: Summary of clinical signs - Female rats

Sl. No	Treatment group	Clinical signs
1	Control Group (n=6)	No Abnormality detected
2	Disease Group (n=6)	No Abnormality detected
3	Test Group (n=6)	No Abnormality detected

Table 25A: Summary of body weight (g) - Male rats

Sl. No	Treatment group	Days				
		0	7	14	21	28
1	Control Group (n=6)	153±3.57	174±6.51	187±7.31	200±8.16	212±9.38
2	Disease Group (n=6)	171±3.44	194±4.43	210±5.56	228±5.89	238±6.44
3	Test Group (n=6)	175±7.81	197±5.95	211±5.83	208±7.36	225±7.89

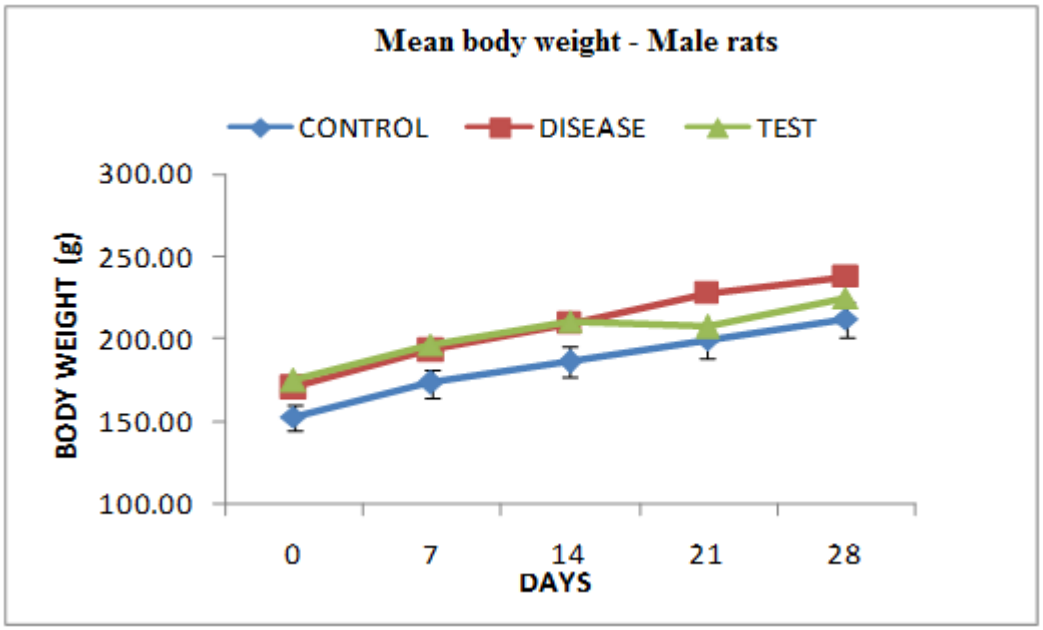

Figure 27. Mean body weight of male rats

Table 25B**:** Summary of body weight **(g)** - Female rats

Sl. No.	Treatment groups	Days				
		0	**7**	**14**	**21**	**28**
1	Control Group (n=6)	155±5.42	162±6.75	170±6.01	168±7.36	176±6.59
2	Disease Group (n=6)	166±3.17	173±3.23	176±2.87	182±4.06	182±3.11
3	Test Group (n=6)	167±2.72	173±3.39	176±3.53	184±4.11	189±3.48

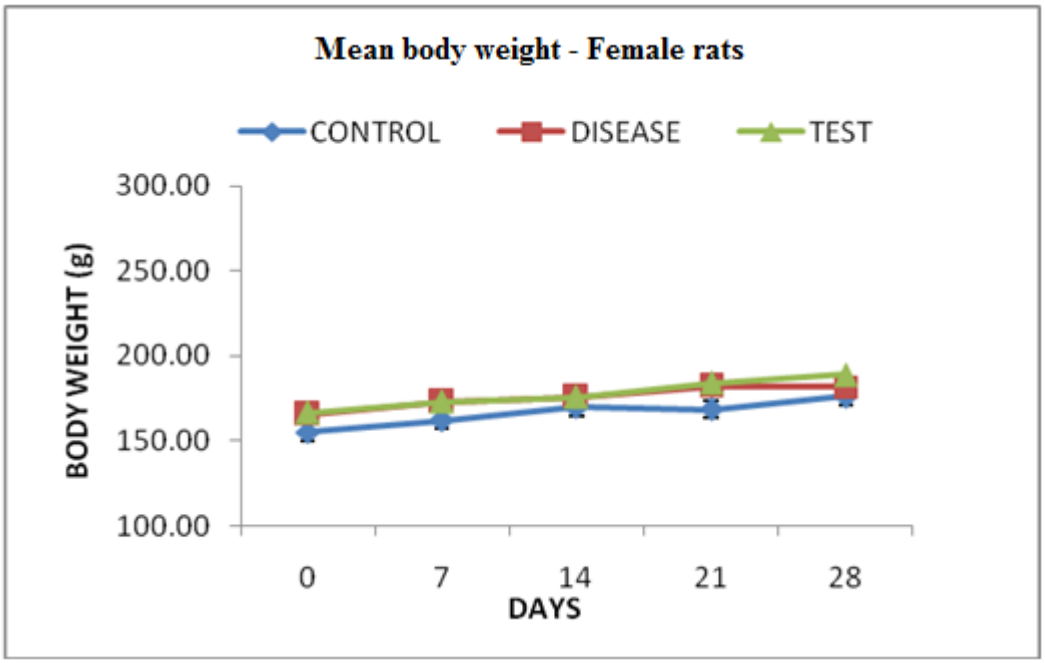

Figure 28. Mean body weight of female rats

Table 26A: Summary of absolute organ weight (g) - Male rats

Sl. No.	Treatment groups	Brain	Lungs	Heart	Liver	Spleen	Kidneys	Adrenals	Testis	Epididymis
1	Control Group (n=6)	1.86±0.03	1.31±0.06	0.72±0.06	7.18±0.48	0.93±0.12	1.55±0.09	0.04±0.00	2.20±0.12	0.83±0.03
2	Disease Group (n=6)	1.86±0.05	1.48±0.10	0.82±0.04	7.84±0.23	0.97±0.09	1.69±0.06	0.03±0.00	2.40±0.16	0.865±0.05
3	Test Group (n=6)	1.95±0.05	1.37±0.07	0.72±0.02	7.02±0.39	0.84±0.08	1.60±0.07	0.04±0.00	1.98±0.27	0.835±0.05

Statistically not significant, P<0.05

Table 26B: Summary of absolute organ weight (g) - Female rats

Sl. No.	Treatment groups	Brain	Lungs	Heart	Liver	Spleen	Kidneys	Adrenals	Ovaries	Uterus
1	Control Group (n=6)	1.77±0.03	1.10±0.05	0.64±0.03	4.78±0.20	0.57±0.10	1.17±0.03	0.05±0.00	0.09±0.01	0.36±0.03
2	Disease Group (n=6)	1.71±0.07	1.20±0.04	0.60±0.02	4.91±0.11	1.00±0.08	1.22±0.03	0.04±0.00	0.10±0.01	0.44±0.05
3	Test Group (n=6)	1.75±0.09	1.04±0.05	0.65±0.02	5.24±0.26	0.67±0.06	1.28±0.03	0.04±0.00	0.09±0.01	0.38±0.04

Statistically not significant, P<0.05

Table 27 A: Summary of haematology data - Male rats

Sl. No.	Treatment groups	RBC/ 10^6/µl	WBC/ 10^3/µl	Differential count (%)			Hb (g/dl)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)	PLT 10^3/µl
				Neutrophils	Lymphocytes	Mixed						
	Normal values	**6.07-10.63**	**5.1-19.9**	**2.6-26.3**	**65.0-96.5**	**0.9-4.9**	**9.1-17.8**	**30.3-60.9**	**48.4-59.8**	**13.8-19.8**	**25.8-33.9**	**500-1300**
1	Control Group (n=6)	7.65 ± 0.20	18.5 ± 1.35	23.8 ± 2.60	72.2 ± 2.79	4.1 ± 0.27	14.2 ± 0.25	44.9 ± 0.51	58.9 ± 1.33	18.53 ± 0.29	31.6 ± 0.31	873.7 ± 16.26
2	Disease Group (n=6)	8.34 ± 0.23	13.3 ± 2.04	24.5 ± 2.39	71.5 ± 2.59	4.0 ± 0.36	14.8 ± 0.31	46.9 ± 0.93	56.30 ± 0.55	17.8 ± 0.32	31.6 ± 0.42	958.5 ± 45.61
3	Test Group (n=6)	7.57 ± 0.33	12.7 ± 1.41	22.3 ± 1.89	73.7 ± 2.07	4.0 ± 0.25	14.0 ± 0.47	44.5 ± 1.67	58.97 ± 1.24	18.6 ± 0.30	31.6 ± 0.41	829.8 ± 70.18

Table 27B: Summary of haematology data - Female rats

Sl. No.	Treatment groups	RBC/ 10^6/µl	WBC/ 10^3/µl	Differential count (%)			Hb (g/dl)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)	PLT 10^3/µl
				Neutrophils	Lymphocytes	Mixed						
	Normal values	**6.07-10.63**	**5.1-19.9**	**2.6-26.3**	**65.0-96.5**	**0.9-4.9**	**9.1-17.8**	**30.3-60.9**	**48.4-59.8**	**13.8-19.8**	**25.8-33.9**	**500-1300**
1	Control Group (n=6)	7.41 ± 0.42	11.1 ± 1.66	24.1 ± 2.83	71.5 ± 2.96	4.4 ± 0.28	13.4 ± 0.57	41.0 ± 1.86	55.5 ± 0.89	18.2 ± 0.31	32.8 ± 0.27	987.2 ± 37.02
2	Disease Group (n=6)	7.73 ± 0.14	12.7 ± 1.34	22.5 ± 4.61	73.4 ± 5.27	4.1 ± 0.71	13.3 ± 0.23	41.6 ± 0.86	53.8 ± 0.48	17.3 ± 0.22	32.1 ± 0.33	889.5 ± 69.90
3	Test Group (n=6)	7.74 ± 0.11	8.6 ± 1.43	14.0 ± 1.64	83.3 ± 1.77	2.8 ± 0.18	14.3 ± 0.12	43.7 ± 0.41	56.5 ± 0.50	18.4 ± 0.23	32.6 ± 0.20	1173.0 ± 36.62

Table 28A: Summary of clinical chemistry data - Male rats

Sl. No.	Treatment groups	Total protein (g/dl)	Albumin (g/dl)	Cholesterol (mg/dl)	SGPT (U/L)	SGOT (U/L)	S. Creatinine (mg/dl)	Glucose (mg/dl)	S. Urea (mg/dl)	Triglycerides (mg/dl)
	Normal values	**4.0-9.0**	**2.3-5.4**	**40-130**	**44.7-186**	**68.6-192**	**0.2-0.75**	**50-170**	**42-82**	**30-162**
1	Control Group (n=6)	6.9±0.17	5.0±0.08	68.0±3.02	63.1±3.74	146.4±13.39	0.73±0.02	92.4±6.26	46.3±1.47	59.7±3.38
2	Disease Group (n=6)	6.0±0.10	5.3±0.08	63.3±3.33	55.2±1.42	149.9±6.25	0.71±0.02	114.0±6.45	47.4±1.19	69.2±6.76
3	Test Group (n=6)	6.4±0.31	4.9±0.20	62.3±3.57	57.5±3.02	145.7±13.03	0.68±0.02	115.1±15.92	48.3±1.71	50.5±8.72

Table 28B: Summary of clinical chemistry data - Female rats

Sl. No.	Treatment groups	Total protein (g/dl)	Albumin (g/dl)	Cholesterol (mg/dl)	SGPT (U/L)	SGOT (U/L)	S. Creatinine (mg/dl)	Glucose (mg/dl)	S. Urea (mg/dl)	Triglycerides (mg/dl)
	Normal values	**4.0-9.0**	**2.3-5.4**	**40-130**	**44.7-186**	**68.6-192**	**0.2-0.75**	**50-170**	**42-82**	**30-162**
1	Control Group (n=6)	7.1±0.11	5.3±0.12	69.5±1.67	64.8±5.65	138.7±11.18	0.72±0.03	82.1±5.22	49.6±2.79	60.3±4.94
2	Disease Group (n=6)	6.7±0.12	4.8±0.07	52.3±3.04	60.7±5.27	150.2±15.01	0.73±0.02	83.1±6.80	50.6±2.51	54.7±6.33
3	Test Group (n=6)	6.7±0.16	5.2±0.12	59.2±2.96	62.2±7.46	137.3±12.30	0.70±0.01	100.7±9.40	51.9±1.89	54.8±4.12

Table 29A: Summary of necropsy findings - Male rats

Sl. No.	Treatment groups	Necropsy findings					
		Heart	Lung	Spleen	Kidneys	Liver	Adrenals
1	Control Group (n=6)	Appears normal with large blood vessels	Non congestive, spongy	Appears normal	Appears normal, with smooth surface	Normal with smooth surface	Appears Normal
2	Disease Group (n=6)	Appears normal with large blood vessels	Non congestive, spongy	Appears normal	Appears normal, with smooth surface	Normal with smooth surface	Appears Normal
3	Test Group (n=6)	Appears normal with large blood vessels	Non congestive, spongy	Appears normal	Appears normal, with smooth surface	Normal with smooth surface	Appears Normal

Table 29B: Summary of necropsy findings - Female rats

Sl. No.	Treatment groups	Necropsy findings					
		Heart	Lung	Spleen	Kidney	Liver	Adrenals
1	Control Group (n=6)	Appears normal with large blood vessels	Non congestive, spongy	Appears normal	Appears normal, with smooth surface	Normal with smooth surface	Appears Normal
2	Disease Group (n=6)	Appears normal with large blood vessels	Non congestive, spongy	Appears normal	Appears normal, with smooth surface	Normal with smooth surface	Appears Normal
3	Test Group (n=6)	Appears normal with large blood vessels	Non congestive, spongy	Appears normal	Appears normal, with smooth surface	Normal with smooth surface	Appears Normal

Table 30A: Summary of histopathology data of brain samples- Control group

Sl. No.	Treatment groups	FINDING
1	Control group- Male rats (n=6)	Brain section shows histology of brain tissue with normal nerve cells and glial tissues.
2	Control group – Female rats (n=6)	Brain section shows histology of brain tissue with normal nerve cells and glial tissues

Table 30B: Summary of histopathology data of brain samples- Disease group

Sl. No.	Treatment groups	FINDING
1	Disease group- Male rats (n=6)	Brain section shows histology of brain tissue with normal nerve cells and glial tissues
2	Disease group – Female rats (n=6)	Brain section shows histology of brain tissue with normal nerve cells and glial tissues

Table 30C: Summary of histopathology data of brain samples- Test group

Sl. No.	Treatment groups	FINDING
1	Test group- Male rats (n=6)	Brain section shows histology of brain tissue with normal nerve cells and glial tissues
2	Test group – Female rats (n=6)	Brain section shows histology of brain tissue with normal nerve cells and glial tissues

7.4.1 Histopathology brain samples:

a. Control group : Male rat

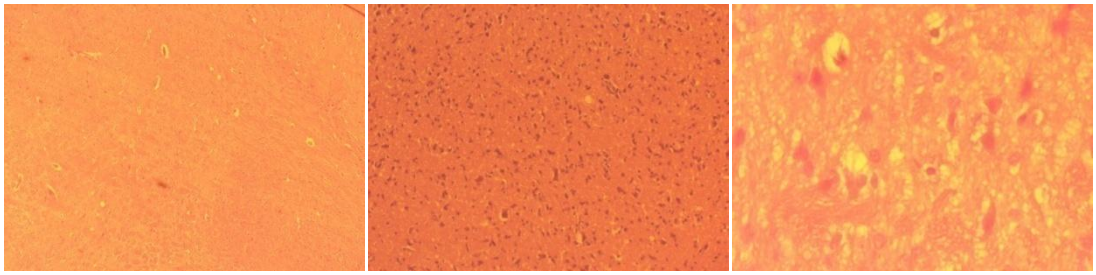

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Figure 29: Microscopic picture of histological plates of brain samples –Control group

b. Control group: Female rat

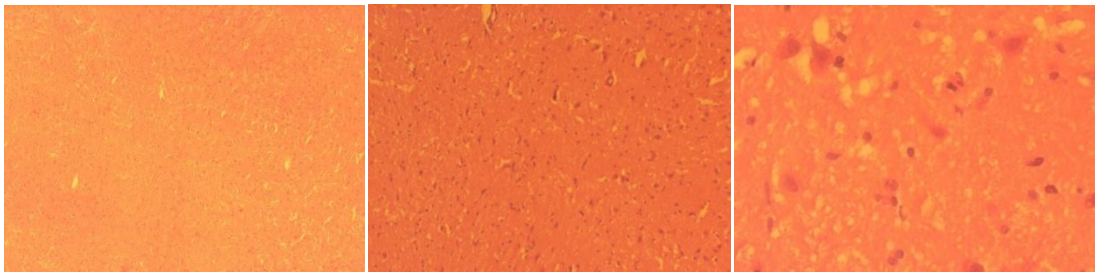

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Figure 30: Microscopic picture of histological plates of brain samples –Control group

c. Disease group :Male rat

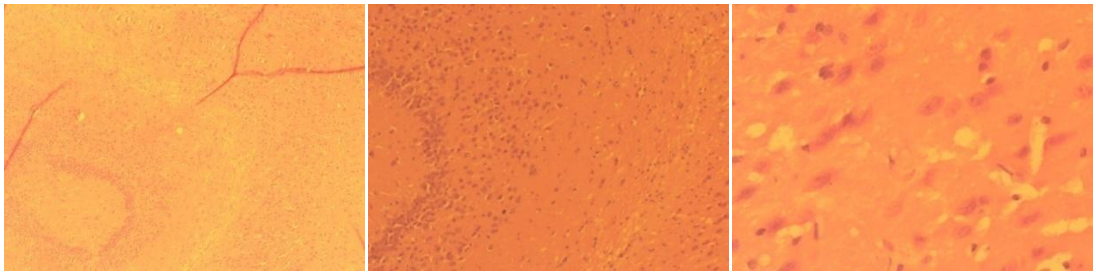

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Figure 31**:** Microscopic picture of histological plates of brain samples – Disease group

d. Disease group :Female rat

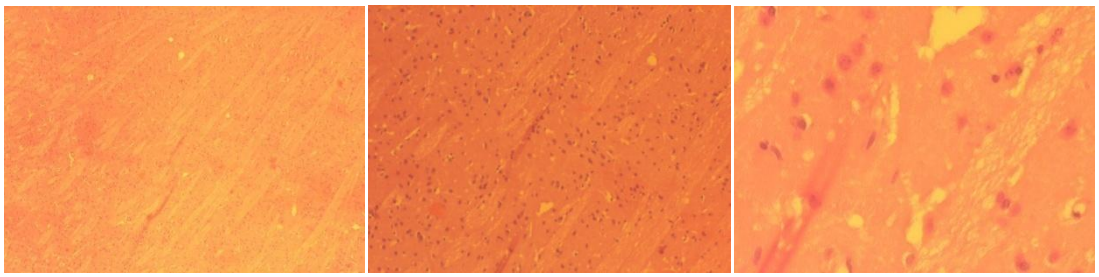

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Figure 32**:** Microscopic picture of histological plates of brain samples –Disease group

e. Test group: Male rat

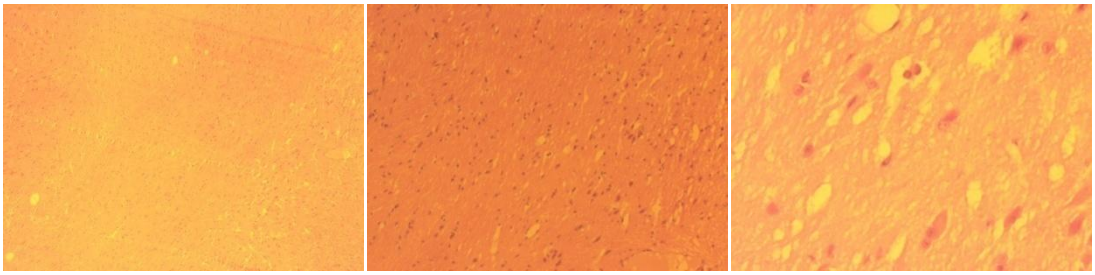

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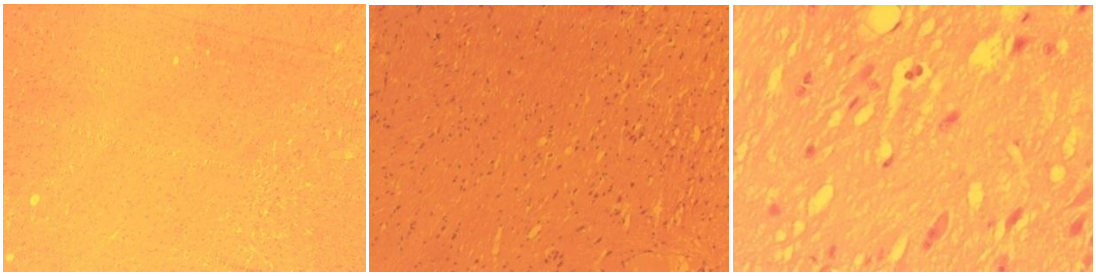
Figure 33: Microscopic picture of histological plates of brain samples- Test group

f. Test group: Female rat



(4X) (10X) (40X)

Figure 34: Microscopic picture of histological plates of brain samples-Test group

Table 31A. Summary of Lipid Peroxidation (LPO) in brain samples – Male rats

Sl. No	Treatment Group	LPO Value (nmol/g fresh weight×10^{-5})
1	Control Group (n=6)	2.65±0.09
2	Disease Group (n=6)	8.41±0.27*
3	Test Group (n=6)	3.24±0.21

*** Statistically significant, P<0.05**

Table 31B. Summary of Lipid Peroxidation (LPO) in brain samples- Female rats

Sl. No	Treatment Group	LPO Value (nmol/g fresh weight×10^{-5})
1	Control Group (n=6)	3.38±0.02
2	Disease Group (n=6)	9.24±0.19*
3	Test Group (n=6)	3.74±0.23

*** Statistically significant, P<0.05**

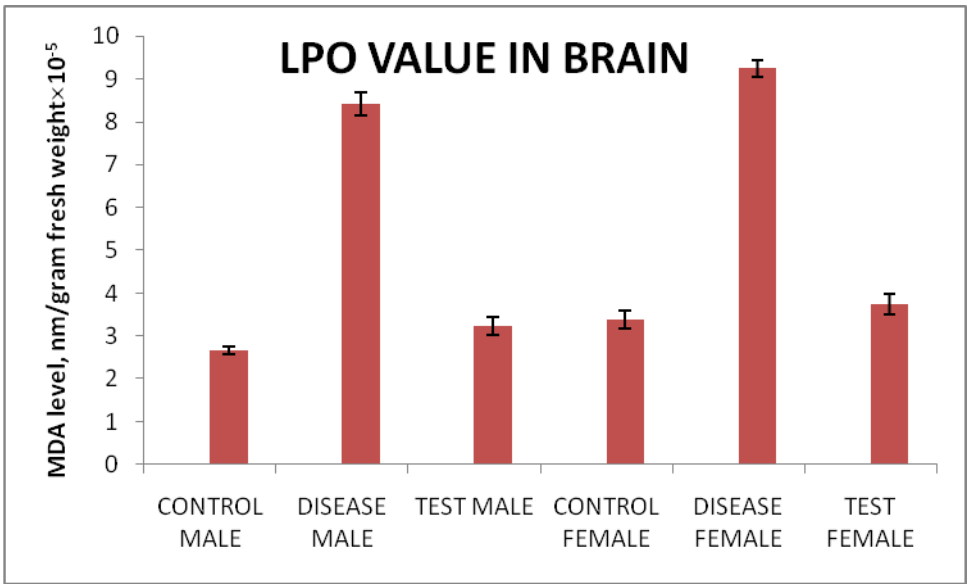


Figure 35A. Summary of Lipid Peroxide (LPO) value in the brain

Table 31C. **S**ummary of Lipid Peroxidation (LPO) in brain samples

Sl.No	Treatment Group	LPO VALUE (nmol/gram fresh weight×10^{-5})
1	Control Group (n=12)	3.0±0.16
2	Disease Group (n=12)	8.8±0.20*
3	Test Group (n=12)	3.5±0.14

*** Statistically significant, P<0.05**

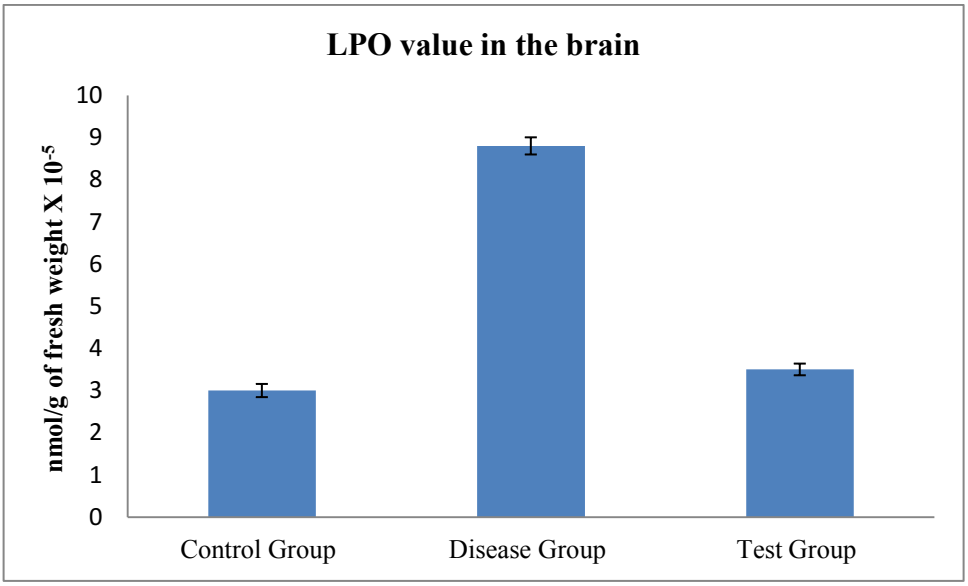


Figure 35B. **S**ummary of Lipid Peroxidation (LPO) in brain samples

Table 32A. Summary of circulating Super Oxide Dismutase (SOD) activity -RBC lysate - Male rats

Sl. No	Treatment Group	SOD VALUE Units/ml of Lysate
1	Control Group (n=6)	915.7±22.78
2	Disease Group (n=6)	401.9±8.82*
3	Test Group (n=6)	902.9±31.52

*** Statistically significant, P<0.05**

Table 32B. Summary of circulating Super Oxide Dismutase (SOD) activity -RBC lysate - Female rats

Sl. No	Treatment Group	SOD VALUE Units/ml of Lysate
1	Control Group (n=6)	826.3±18.03
2	Disease Group (n=6)	407.0±12.37*
3	Test Group (n=6)	801.9±20.59

*** Statistically significant, P<0.05**

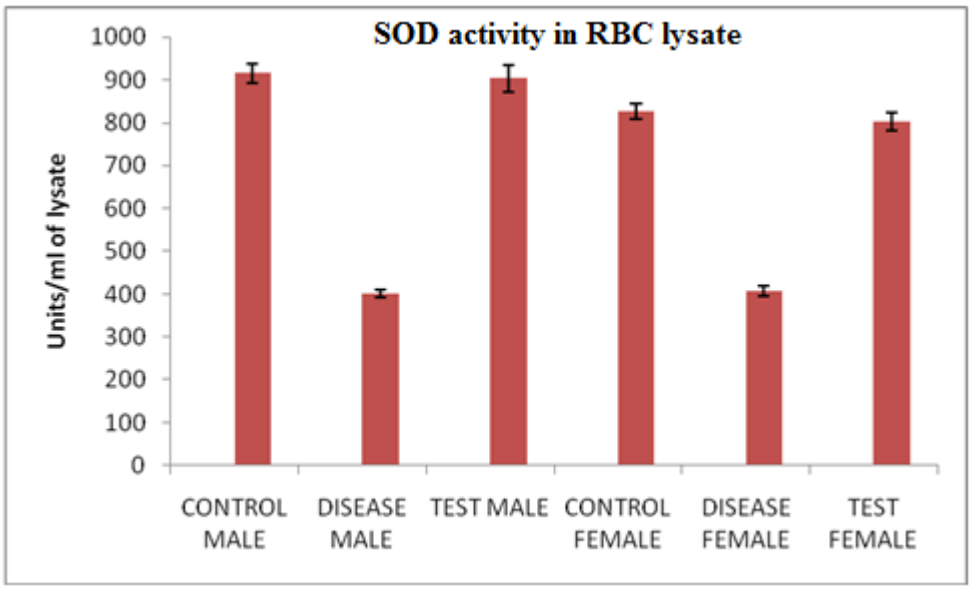


Figure 36A. Summary of circulating Super Oxide Dismutase (SOD) activity in RBC lysate

Table 32C. Summary of circulating Super Oxide Dismutase (SOD) activity –RBC lysate

Sl. No	Treatment Group	SOD Value Units/ml of Lysate
1	Control Group (n=12)	871.0±19.32
2	Disease Group (n=12)	404.4±7.28*
3	Test Group (n=12)	852.4±23.54

*** Statistically significant, P<0.05**

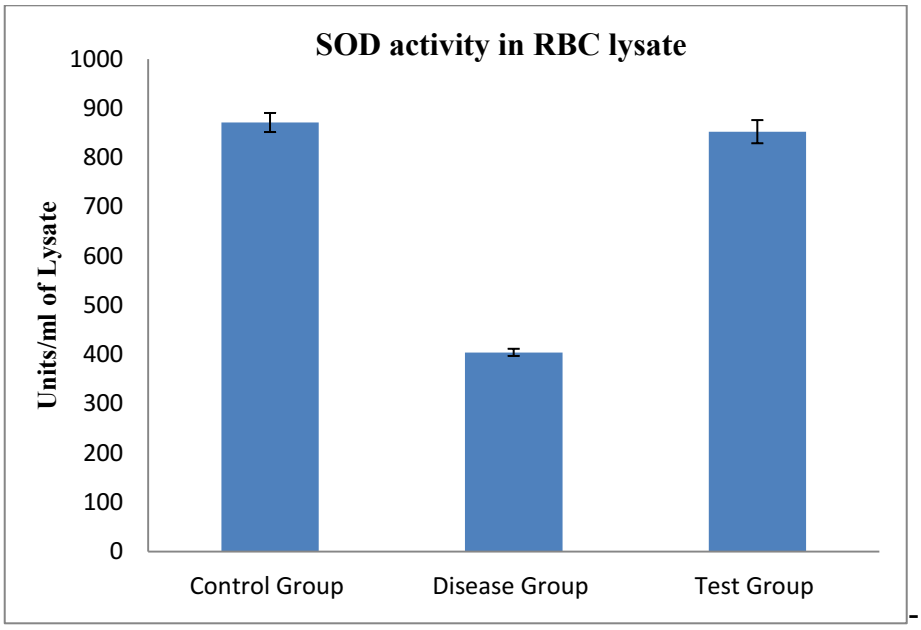


Figure 36B. Summary of circulating Super Oxide Dismutase (SOD) activity in RBC lysate

Table 33A: Summary of circulating Super Oxide Dismutase (SOD) activity in blood plasma - Male rats

Sl. No	Treatment Group	SOD VALUE Units/ml of Plasma
1	Control Group (n=12)	422.1±14.31
2	Disease Group (n=12)	234.6±30.19*
3	Test Group (n=12)	425.7±13.83

* Statistically significant, P<0.05

Table 33B: Summary of circulating Super Oxide Dismutase (SOD) activity in blood plasma - Female rats

Sl. No	Treatment Group	SOD VALUE Units/ml of Plasma
1	Control Group (n=12)	449.0±23.21
2	Disease Group (n=12)	255.6±18.69*
3	Test Group (n=12)	443.8±23.53

* Statistically significant, P<0.05

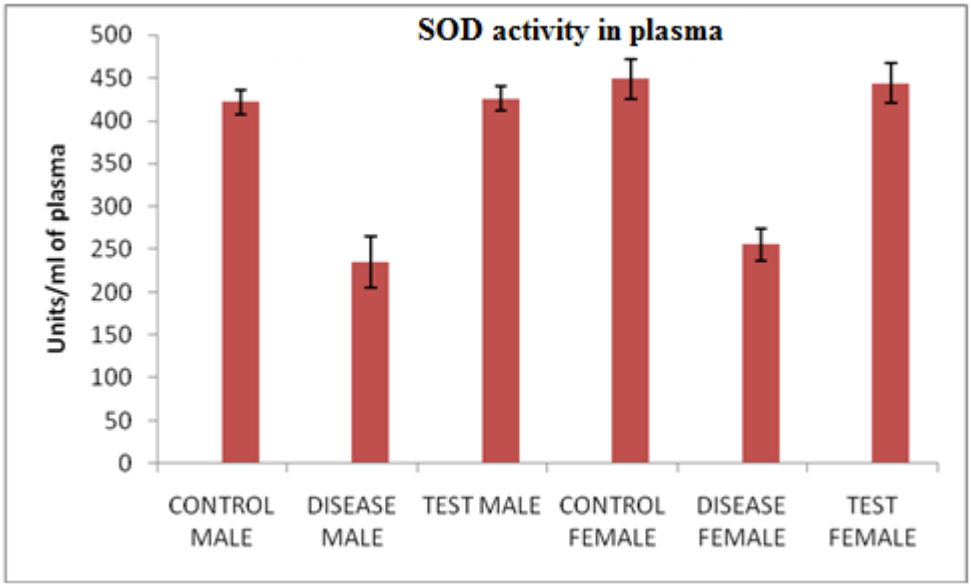


Figure 37A. Summary of circulating Super Oxide Dismutase (SOD) activity in blood plasma

Table 33C: Summary of circulating Super Oxide Dismutase (SOD) activity in blood plasma

Sl. No	Treatment Group	SOD Value Units/ml of Plasma
1	Control Group (n=12)	435.5±13.62
2	Disease Group (n=12)	245.1±17.22*
3	Test Group (n=12)	434.8±13.30

* Statistically significant, P<0.05

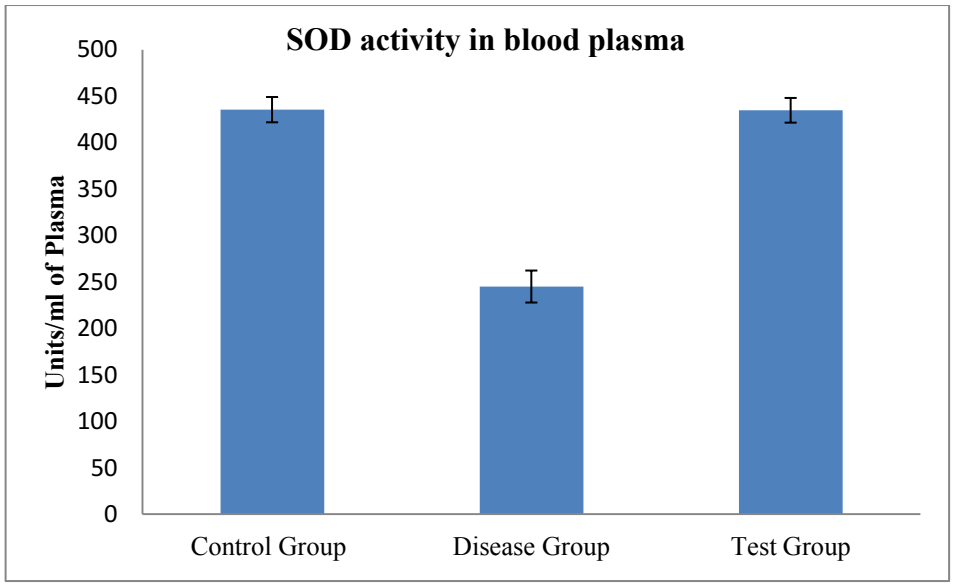


Figure 37B. Summary of circulating Super Oxide Dismutase (SOD) activity in blood plasma

Table 34A: Summary of Super Oxide Dismutase (SOD) activity in the brain- Male rats

Sl. No	Treatment Group	SOD Activity Units/gram fresh weight
1	Control Group (n=6)	797.7±29.71
2	Disease Group (n=6)	430.5±34.03*
3	Test Group (n=6)	815.6±30.90

* Statistically significant, P<0.05

Table 34B: Summary of Super Oxide Dismutase (SOD) activity in the brain- Female rats

Sl. No	Treatment Group	SOD Activity Units/gram fresh weight
1	Control Group (n=6)	817.8±15.51
2	Disease Group (n=6)	436.1±14.79*
3	Test Group (n=6)	828.8±11.83

* Statistically significant, P<0.05

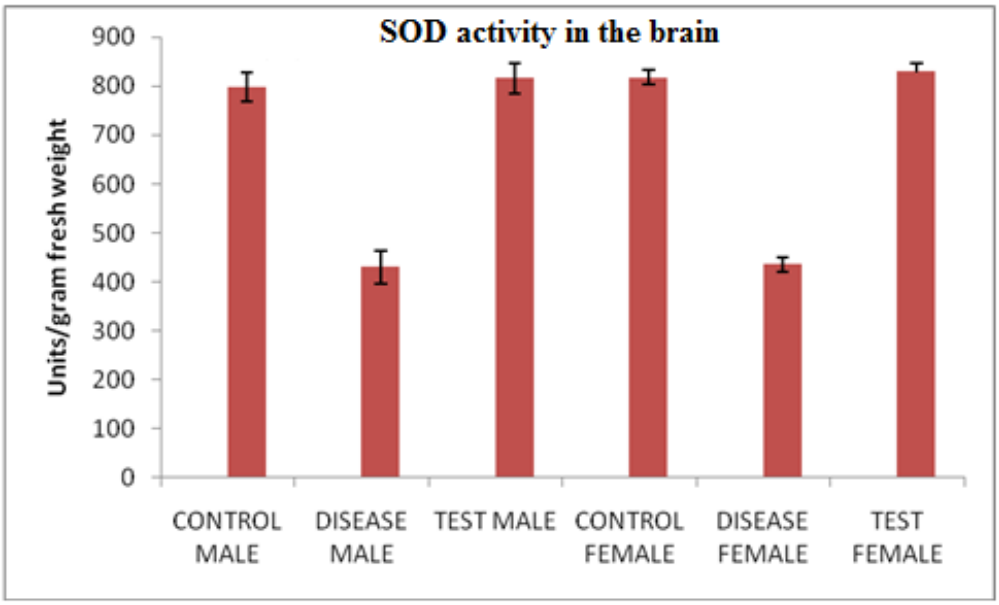


Figure 38A. Summary of brain Super Oxide Dismutase (SOD) activity in the brain

Table 34C: Summary of Super Oxide Dismutase (SOD) activity in the brain

Sl. No	Treatment Group	SOD Activity Units/gram fresh weight
1	Control Group (n=12)	807.7±16.26
2	Disease Group (n=12)	433.4±17.71*
3	Test Group (n=12)	822.2±15.90

* Statistically significant, P<0.05

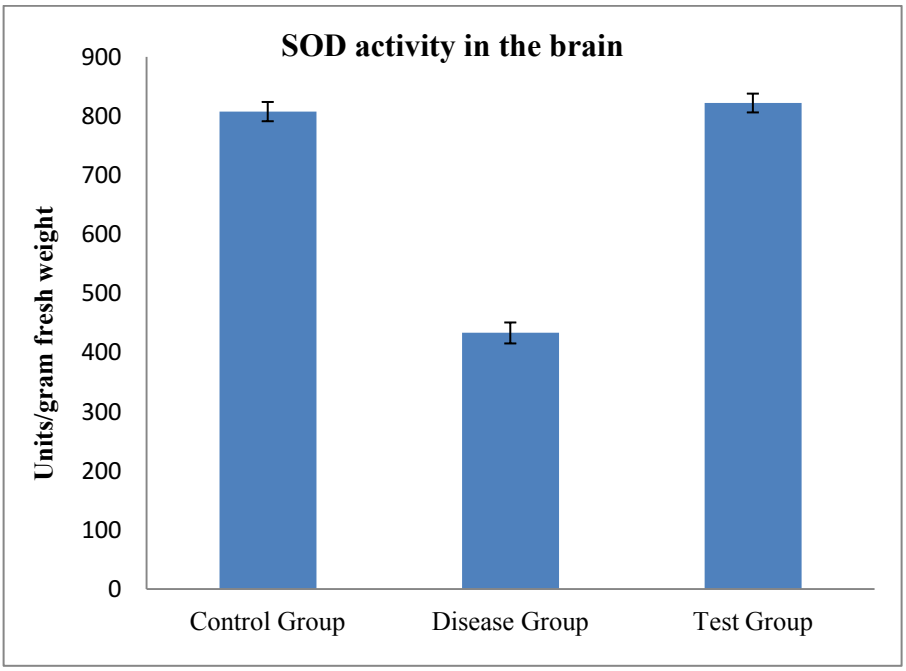


Figure 38B. Summary of brain Super Oxide Dismutase (SOD) activity in the brain

Table 35A: Summary of protein phosphatase 2A (PP2A) data in the brain - Male rats

Sl. No.	Treatment group	PP2A Activity pmol/min/µg of protein
1	Control Group (n=6)	3.8±0.20
2	Disease Group (n=6)	0.6±0.05*
3	Test Group (n=6)	3.4±0.20

* Statistically significant, P<0.05

Table 35B: Summary of protein phosphatase 2A (PP2A) activity in the brain - Female rats

Sl. No.	Treatment group	PP2A Activity pmol/min/µg of protein
1	Control Group (n=6)	3.7±0.3
2	Disease Group (n=6)	0.9±0.09*
3	Test Group (n=6)	3.3±0.2

* Statistically significant, P<0.05

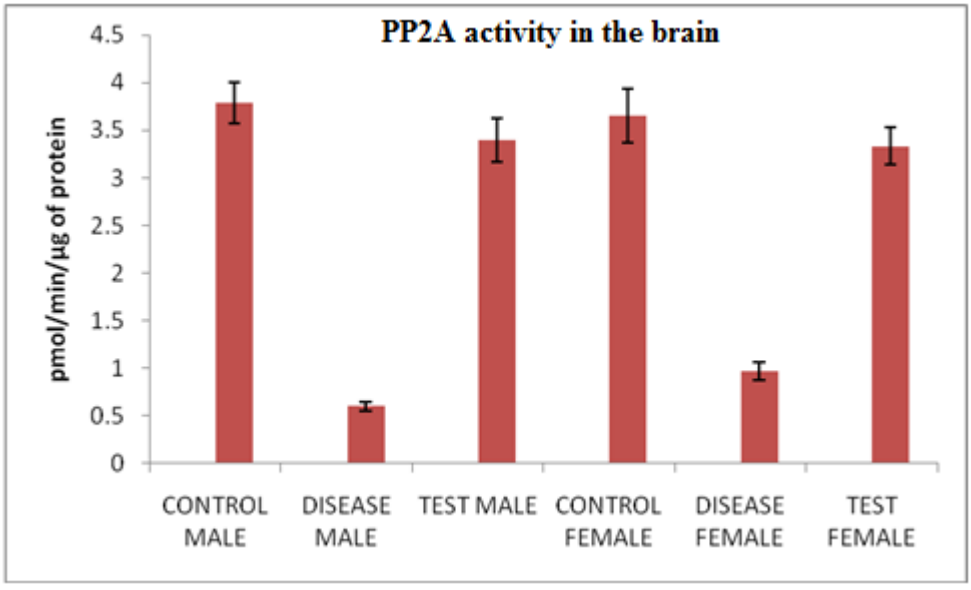


Figure 39A. Summary of protein phosphatase 2A (PP2A) activity in the brain

Table 35C: Summary of protein phosphatase 2A (PP2A) activity in the brain

Sl. No	Treatment Group	PP2A ACTIVITY pmol/min/µg of protein
1	Control Group (n=12)	3.7±0.17
2	Disease Group (n=12)	0.8±0.07*
3	Test Group (n=12)	3.4±0.14

* Statistically significant, P<0.05

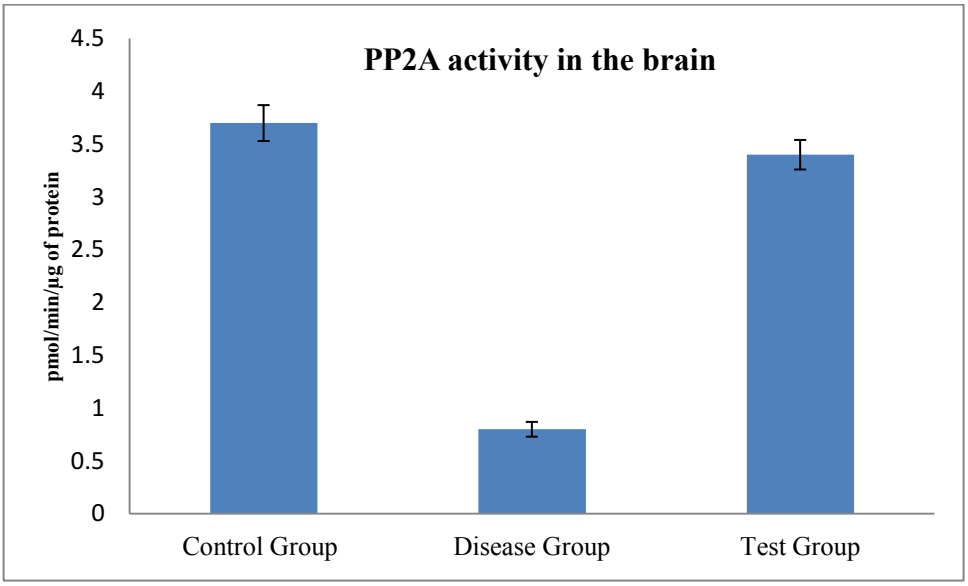


Figure 39B. Summary of protein phosphatase 2A (PP2A) activity in the brain

7.5 Summary:

The neuroprotective efficacy of BMC in aluminium chloride-induced Alzheimer's disease model was evaluated in Sprague-Dawley rats after an oral dose of 500 mg/kg (BMC equivalent) body weight per day (in two divided doses) for 28 days. Alzheimer's disease was induced in Sprague-Dawley rats by intraperitonial administration of Aluminium chloride. In this animal model, various parameters were investigated which includes, estimation of lipid peroxidation (LPO) in the brain, superoxide dismutase (SOD) activity in the brain, circulating superoxide dismutase activity in blood and protein phosphatase 2A (PP2A) activity in the brain. In addition, histopathology of the brain was performed in order to locate the probable sites of degeneration. BMC, at given dose, showed potent neuroprotective activity as evidenced from the results.

Oral administration of BMC significantly ($P<0.05$) reduced Lipid Peroxidation (LPO) as compared to the disease group (3.5±0.14 and 8.8±0.20 nmol/g for BMC and Disease group, respectively). Lipid peroxidation was high in the disease group implicating increased oxidative stress upon administration of aluminum chloride. Lipid peroxidation in BMC group was comparable to control group (3.5±0.14 and 3.0±0.16 nmol/g for BMC and Control group, respectively) showing the antioxidant activity of BMC in *in vivo* conditions.

Also, BMC significantly ($P<0.05$) increased the levels of SOD activity in brain (822.2±15.90 units/g), plasma (434.8±13.30 units/g) and in RBC (852.4±23.54 units/g) as compared to disease group (brain; 433.4±17.71 units/g, plasma; 245.1±17.22 units/g and RBC; 404.4±7.28 units/g). No significant difference was observed for SOD activity between BMC group and control group (brain; 807.7±16.26 units/g, plasma; 435.5±13.62 units/g and RBC; 871.0±19.32 units/g). The SOD activity in BMC group was found to be comparable to that of control group indicating antioxidant property of the BMC in the developed animal model. Administration of BMC orally at the dose of 500 mg/kg body weight was found to combat oxidative stress produced by accumulation of aluminum through up regulating antioxidant enzymes. These data were in line with data from *in vitro* studies, where BMC was proven to be a potent antioxidant.

Also, in BMC group, PP2A activity in the brain (3.4±0.14 pmol/min/µg of protein) was significantly ($P<0.05$) increased when compared to disease group (0.8±0.07 pmol/min/µg of protein). However, PP2A activity in the brain of the BMC group was comparable to control group (3.7±0.17 pmol/min/µg of protein). Increased PP2A activity in BMC group might lead to the reduced hyperphosphorylation of tau and consequently reduced neurofibrillary tangles (Walton, 2007) thus decreasing neurological damage caused by aluminum chloride upon treatment with BMC. Nevertheless, BMC was found to be an effective neuroprotective compound in aluminium chloride-induced Alzheimer's disease rat model.

There was no significant change in body weight in all the three groups. No major behavioral changes were observed in any of the groups. However, in test group, after dosing of BMC, urine showed reddish yellow color in all the animals and also dark black colored feces in 4^{th} and 6^{th} animal from male test group. This color may be due to the BMC and its metabolite. No abnormalities were detected on necropsy in the animals of all three groups. Clinical biochemistry values and haematology values were within normal range in all the groups. These data also shows that BMC is safe at the dose tested.

In conclusion, the BMC was found to be a potent neuroprotective agent and safe in aluminum chloride-induced Alzheimer's disease rat model.

Chapter-8

8. Discussion and conclusions:

8.1 Discussion:

Alzheimer's disease is an irreversible, progressive brain disease that slowly destroys memory and cognitive skills (Shankar and Walsh, 2009; Castro *et al.,* 2010). It is a multifactorial disease involving several risk factors such as age, senile plaques, neurofibrillary tangles, oxidative stress, metal toxicity, BACE1 activity, lower levels of neurotransmitters and genetic factors (Gracy *et al.,* 1999; Morrison *et al.,* 2005; Castro *et al.,* 2010; Knopman *et al.,* 2010; Bajda *et al.,* 2011). Alzheimer's disease is characterized by the deposition of β-amyloid plaques and neurofibrillary tangles in the brain, which are toxic to neuronal cells. Aβ peptide, a major component of Aβ plaques, is a product of proteolytic cleavage of amyloid precursor protein (APP) (Bajda *et al.,* 2011). Amyloid precursor protein is a transmembrane protein which is enzymatically cleaved by 2 different proteolytic enzymes (BACE1 and γ-secretase) in sequential order, which results in the production of $A\beta_{1-40}$ and $A\beta_{1-42}$ fragments. BACE1 is one of the major and validated targets for the development of therapeutic interventions for the treatment of Alzheimer's disease (Strooper, 2010). There are no therapeutic interventions available for the complete cure of Alzheimer's disease. Currently, available drugs are symptomatic in nature and can only delay the onset of the disease or decrease the symptoms associated with the disease (Cedergren *at al.,* 2007; Gotz and Ittner, 2008). Although, the available drugs are efficient in reducing the disease burden to some extent, there are side effects associated with these drugs, thus limiting their long-term usage. Hence, there is urgent need for the development of effective and safe drug for the treatment of Alzheimer's disease. In current drug discovery and development process, most of the synthetic drugs fail during their development stage either due to lack of efficacy or due to toxicity or both. The possible alternative solution to the present day synthetic drugs would be compounds

(phytochemicals) from natural sources. Compounds from plants are used for human therapeutic applications from time immemorial and are found to be safe for human applications apart from being effective. Many of the natural compounds are known to have neuroprotective and/or memory enhancing activity.

The objective of this doctoral research work was mainly aimed at finding a potential therapeutic lead from natural source for therapeutic application in Alzheimer's disease. The key objective of this study was to screen natural compounds, against one of the validated Alzheimer's disease drug target called BACE1 in *in silico* studies and evaluate the efficacy further in *in vitro* and *in vivo* animal model study, for potential therapeutic application in Alzheimer's disease.

8.1.1. Short-listing natural compounds for *in silico* screening;

Natural compounds such as phytochemicals are well proven for their diverse biological activity. Phytochemicals from several plants were identified and explored extensively as a rich source of lead compounds for therapeutic application (Tariq and Reyaz, 2013). Many of such compounds from natural resources are known to have neuroprotective and/or memory enhancing activity, which can be explored further for therapeutic application in brain diseases. Hence, one of the objectives of this study is to identify such plants/compounds with neuroprotective and memory enhancing property, and elucidate its site of activity using *in silico* tools. As a first part of the study, a thorough literature review phytochemicals and their metabolites with neuroactive applications were short-listed for *in silico* and *in vitro* studies.

Currently, 25% of the world's prescribed drugs have their origin from plants and morphine was the first drug to be isolated from the plant and introduced into the market. The history of using plants, its extracts, purified compounds and their derivatives for several thousands of years demonstrates the safety and efficacy of the plant based drugs (Sripathy *et al.,* 2015). Phytochemicals with neuroprotective and memory enhancing properties through diverse mode of action including antioxidant and anti-inflammatory properties, acetyl choline esterase inhibitory activity, prevention of Aβ formation and deposition, β-secretase

enzyme inhibition *etc* were short-listed. Totally 47 compounds were short-listed based on literature search. The compounds short-listed belonged to the group of Flavonoid (14 compounds), Phenolic compounds (6 compounds), Stilbenoid (1 compound), Vanilloids (1 compound), alkaloids (7 compounds), Quinones (3 compounds), Terpenes (3 compounds), Phytoestrogen (1 compound), Organic compounds (4 compounds), Steroids (1 compound), amino acids (1 compound), Carotenoids (2 compounds), saturated cyclic acids (1 compound) and Glucosinolates (1 compound). The short-listed phytochemicals were screened using *in silico* tools as detailed in Chapter 4.

8.1.2 *In silico* screening of natural compounds:

In *in silico* studies, all the short-listed phytochemicals were evaluated for their binding affinity towards BACE1 using Molecular docking tool called AutoDock version 4.0. Molecular docking is one of the *in silico* tools used for screening compounds to differentiate between good binders and non-binders of target protein (Seeliger and Groot, 2010). The identification of lead molecules showing activity against drug target has been given high importance in early stages of drug discovery to avoid drug failure in the later stages. In the present research work, AutoDock.4 was used for molecular docking to predict the binding energy of phytochemicals (ligand) towards BACE1 enzyme (target protein). AutoDock utilizes Force Field (FF) scoring function, which are developed based on physical atomic interactions, including van der Waals (VDW) interactions, electrostatic interactions and bond stretching/bending/torsional forces (Huang *et al.,* 2010). As per Wang *et al.,* AutoDock had 62% success rate in scoring protein ligand complex (Huang *et al*., 2010). In the present study, the binding energy of short-listed phytochemicals against BACE1 ranged from -5.16 to -21.41 kcal/mol and most of the compounds had binding energy lesser than -13 kcal/mol. This may be due to the structural similarities of phytochemicals among the compounds selected. Lesser binding energy indicates better binding affinity of ligand towards target protein (Barman and Prabhakar, 2014) indicating properties of good drug candidate. Based on the *in silico* binding energy, available safety data, one of the widely studied compound, Curcumin and its natural metabolite, BMC, were short-listed for further studies due to their wide interest in developing as a drug for the

treatment of Alzheimer's disease. Curcumin is one of the phytochemical which is widely studied for its application in the prevention and treatment of Alzheimer's disease. In this research work, Curcumin was used as a reference compound for comparing the efficacy of BMC. Curcumin was found to cross blood brain barrier and exerts its neuroprotective activity. In a study, Curcumin treatment for six months was found to be effective in decreasing the level of β- Amyloid peptides and inflammatory cytokines in transgenic mice harboring Swedish mutation on amyloid precursor protein (APPsw) (Ringman *et al.,* 2012). Safety of the curcumin is studied widely and proven to be safe for human consumption. The oral LD_{50} value for curcumin was found to 2g/kg body weight in mice and oral LD_{50} for curcumin extract was found to be 12.2g/kg body weight. Curcumin was found to be a very potent antioxidant and anti-inflammatory agent and the main mechanism is via down regulation and inhibition of proinflammatory agents (Kohli *et al.,* 2005). Comparing the efficacy of BMC with well studied Curcumin would definitely help in predicting BMC potential as a therapeutic intervention in Alzheimer's disease. The binding energy for BMC was found to be -17.18 kcal/mol and for Curcumin, it was found to be -14.33 kcal/mol indicating good affinity for BACE1 enzyme. BMC (-17.18 kcal/mol) was found to have lesser binding energy towards BACE1 than its reference and parent compound Curcumin (-14.33 kcal/mol), indicating better drug properties than Curcumin. BMC and Curcumin were further evaluated for drug likeness and drug score using Molsoft and Osiris molecular property evaluation tools.

As per the molecular property analysis using Molsoft and Osiris, Curcumin and BMC were found to have good drug score of being potential drug candidates. Positive value for drug score in this drug likeness software indicates that the screened molecule has properties to be potential drug candidate (Ferdous *et al.,* 2013). Although BMC had a negative drug likeness score (-0.82), which was comparatively lesser than Curcumin (0.35), its drug score (0.43) was slightly higher than Curcumin (0.39). Lipinski's Rule of Five predicts that, when there is more than 5 hydrogen bond donor, more than 10 hydrogen bond acceptors, more than 500 da molecular weight and LogP value of more than 5 in a drug molecule, the drug is predicted to have poor bioavailability. Both Curcumin and BMC were in compliance with Lipinski's Rule of five and hence might have better absorption and bioavailability profile.

Molecular docking studies give a predictive analysis for a compound to be a potential drug based on molecular properties. But, there are other factors (Plasma binding protein properties, pKa, metabolism in terms of CYP450 and bioavailability) to be studied to know that the compound is pharmacologically active. Importantly, CNS drug has to cross the BBB to show its biological activity. One has to study all the parameters for a drug candidate (Mishra *et al*., 2003).

Overall, results from the *in silico* study confirmed that BMC has properties of being a potential drug candidate in Alzheimer's disease therapeutic application. However, this has to be further evaluated in *in vitro* and *in vivo* studies before considering BMC for further development as a drug.

8.1.3 *In vitro* evaluation of BMC and Curcumin

Subsequent to *in silico* evaluation, BMC and Curcumin were further evaluated in *in vitro* studies for antioxidant activity, anti-inflammatory activity and BACE1 inhibitory activity. Amyloidogenic pathway is linked to increased oxidative stress and inflammation (Dyall, 2010). Amyloid β peptide has been shown to induce free radical production (Varadarajan *et al.,* 1999) as evidenced by oxidative stress and oxidative modifications of proteins, lipids and DNA in Alzheimer's disease patient's brain (Butterfield *et al.,* 2001). In *in vitro* studies, BMC was found to have superior antioxidant activity compared to its parent compound Curcumin and standard reference compound Vitamin C. It was found to have 5.4 times lower IC_{50} value as compared to Curcumin and 4.9 times lower IC_{50} value as compared to Vitamin C in DPPH radical scavenging assay. In ABTS radical scavenging assay, BMC was found to have 4.1 times lower IC_{50} value when compared to both Curcumin and Vitamin C. The IC_{50} value of BMC was 14.7 and 1.1 times lower when compared to IC_{50} values of Curcumin and Vitamin C, respectively in superoxide radical scavenging assay. In FRAP assay, EC_{50} of BMC was 5.8 times lower than that of Curcumin and 1.7 times lower than that of Vitamin C. Overall, antioxidant activity of BMC was found to be superior to Curcumin and Vitamin C in all tested antioxidant assays. These results were in line with the previous research, where the antioxidant activity of BMC was found to be superior to Curcumin (Abas *et al.,* 2006; Ravindran *et al.,* 2010). The

antioxidant activity of BMC as determined by DPPH method in the current research study was 2.66 μM, which was almost two times lower than the reported results for BMC (5.8±3.8 μM) (Abas *et al.,* 2006). In Alzheimer's disease, oxidative stress is one of the causative factor for cognitive impairment (Keller *et al.,* 2005) followed by inflammatory response (Dyall, 2010). *In vitro* studies indicate that BMC can alleviate the oxidative stress and was also found to be better than curcumin in antioxidant activity.

Inflammatory reactions are strongly associated with Alzheimer's disease pathology (Joshi *et al,* 2015). Chronic inflammation of the nerve cells is one of the important pathophysiological changes seen in Alzheimer's disease. Major inflammatory changes were found to be microgliosis, astrocytosis and induction of pro-inflammatory cytokines which accompany the Aβ plaques in Alzheimer's disease (Mishra *et al.,* 2008). Higher level of tissue pro-inflammatory cytokines such as interleukins-1 β (IL-1β), tumor necrosis factor α (TNFα), and interferon γ (IFNγ) were found to increase the β-amyloid peptide and tau phosphorylation (Dyall, 2010; Joshi *et al*., 2015). IL-1 induces oxidative stress, causing lipid peroxidation and activation of microglial cells to produce inflammatory cytokines. IL-1 also increases the synthesis of APP (Dyall, 2010). Anti-inflammatory drugs were found to be helpful in Alzheimer's disease on long term usage (Mishra *et al.,* 2008) but their long term usage is associated with side effects. Another enzyme, 5-lipoxygenase (5-LOX), which produces pro-inflammatory leukotrienes, is known to be associated with Alzheimer's disease. In an *in vitro* study, 5-LOX knockout cells were shown to have reduced production of Aβ. A similar finding was seen in transgenic mice with 5-LOX knockout. Zileuton, a selective inhibitor of 5-LOX significantly reduced the Aβ deposition in the brains of transgenic mice (Tg2576), indicating selective inhibitor of 5-LOX as a novel therapeutic opportunity in the drug development for Alzheimer's disease (Chu *et al.,* 2011). Also, 5-LOX inhibition is associated with improvement in cognitive functions (Joshi *et al.,* 2015). Hence, one of the objectives of *in vitro* studies in the present research work was to evaluate the short-listed compounds for anti-inflammatory activity.

In the *in vitro* studies, BMC was found to be potent anti-inflammatory agent when compared to Curcumin. BMC inhibited the pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β with IC_{50} value of 88.27 μg/ml, 119.9 μg/ml and 26.94 μg/ml, respectively in

LPS induced whole blood and the values were slightly lesser than IC_{50} value of Curcumin. IC_{50} values of Curcumin and BMC in the inhibition of 5-Lipoxygenase were found to be 27.47 µg/ml and 6.58 µg/ml respectively. Thus, BMC was found to be 4.5 times more potent inhibitor of 5-LOX than curcumin. This study reconfirms the results from previous study carried out by Ravindran *et al.,* where BMC was found to be more potent anti-inflammatory compound than Curcumin (Ravindran *et al.,* 2010). In several studies, Curcumin was found to be very potent antioxidant and anti-inflammatory agent, and the main mechanism is via down regulation and inhibition of proinflammatory agents (Kohli *et al.,* 2005). In animal studies, Curcumin was also found to cross blood brain barrier. In a study, Curcumin treatment for six months was found to be effective in decreasing the level of Aβ peptides and inflammatory cytokines in transgenic mice harboring the APP Swedish mutation (APPsw) (Ringman *et al.,* 2012). Curcumin was also proven to be efficient in combating aluminium induced cognitive dysfunction and oxidative damage in rats (Ramachandran *et al.,* 2013). In current study, BMC was found to be potent antioxidant and anti-inflammatory agent compared to clinically proven Curcumin. Since, oxidative stress and inflammation are the hallmark of pathophysiology of Alzheimer's disease, BMC could be one of the best candidates for therapeutic intervention for Alzheimer's disease. Considering, earlier literature data on Curcumin and current study findings on BMC, one can logically infer that, BMC is probably a potential drug candidate than Curcumin in Alzheimer's disease management, which further needs to be evaluated in *in vivo* studies. Hence, BMC was further evaluated for neuroprotective efficacy in *in vivo* animal model study.

Subsequent to the antioxidant and anti-inflammatory evaluation, BMC was studied for its inhibitory activity against BACE1. BACE1 is the first enzyme to cleave Met671-Asp672 amide bond of amyloid precursor protein (APP) in the amyloidogenic pathway leading to the production of Aβ peptide (Barman and Prabhakar, 2014). BACE1 is an important and attractive target for the development of drug for the treatment of Alzheimer's disease. The development of BACE1 inhibitors was actively perused in recent years (Arun *et al.,* 2008). In the current research work, the IC_{50} value of BMC for the inhibition of BACE1 was found to be 1.471 µg/ml and this result confirms effective inhibition of BACE1. This *in vitro*

study result confirmed the results from *in silico* studies indicating correlation between *in silico* and *in vitro* results.

8.1.4 Formulation development for improving the aqueous solubility of BMC:

Phytochemicals such as Curcumin are hydrophobic in nature, and are insoluble in gastric and intestinal fluids leading to their poor bioavailability. Many of the phytochemicals were found to have potential therapeutic benefits, but could not be developed as successful drug due to their poor aqueous solubility and bioavailability. Although, they perform very well in *in vitro* studies, they fail to perform in *in vivo* studies due to their poor pharmacokinetic profile. About 70% of the new drug candidate showed poor solubility limiting the *in vivo* bioavailability. These drug candidates and phytochemicals would show improved oral bioavailability if their aqueous solubility is improved (Khadka *et al.,* 2014). Hence, before conducting the *in vivo* efficacy study, it was necessary for developing a formulation for improving the aqueous solubility of BMC. There are several techniques such as liposomes, emulsions, solid lipid nanoparticles, nanostructured lipid carriers, micelles and poly(lactic-co-glycolic acid) nanoparticles which were adopted for enhancing the aqueous solubility and oral bioavailability of poorly soluble drugs (Wang *et al*., 2014). Self emulsifying drug delivery system (SEDDS) has been widely used for increasing the solubility and bioavailability of drug with great success. SEDDS are isotropic mixture of lipids, surfactants and co-solvents, which forms oil in water emulsion in water. Nonionic surfactants such as polysorbates are preferred for SEDDS due to their low CMC. SEDDS are more suitable for the drugs with LogP value in the range of 2 and 4 (Sarpal *et al.,* 2010). The LogP value for BMC was found to be 2.44 and was in the range specified by Sarpal *et al.,* 2010, for SEDDS. Hence, SEDD formulation was developed for BMC using polysorbate 80 and PEG400, which forms oil in water microemulsion in aqueous solutions. Polysorbate 80 was selected due to its acceptance in food as an emulsifier with an ADI limit of 0-25 mg/kg body weight (Joint FAO/WHO Expert Committee on Food Additives (JECFA)). It is a hydrophilic emulsifier with HLB value of 15, which forms oil in water

emulsion in water. PEG 400 was used as co-solvent in the formulation. Use of combinations of surfactants, and co-solvent enhances the solubility of the drug to a larger extent (Liu and Guo, 2007) and this justifies the use of PEG 400 in the BMC formulation. The total HLB value for the formulation was calculated to be 11.28, which confirms that the formulation forms oil in water emulsion in water (Friberg, 2003). The concentration of BMC in the formulation was found to be 5.67% as analyzed by HPLC. The developed BMC formulation was found to have good solubility in water, confirming the successful achievement of the objective. Generally, the SEDD formulations form microemulsion in water with the particle size ranging from 100 to 250 nm (Sarpal *et al.,* 2010). The particle size of BMC formulation in water was 254 nm (Figure 25), which was slightly above the range provided by Sarpal *et al*. Through TEM study, it was found that the particles were of spherical in nature. Also, it was found to have good cellular uptake as evidenced by cellular uptake study in Caco2 cell lines. Developed BMC formulation was used for the evaluation of its efficacy in aluminium chloride-induced Alzheimer's disease model.

8.1.5 Evaluation of efficacy of BMC in *in vivo* animal model study:

Several animal models including primates, dogs, ageing rats, chemical induced rodents, genetically modified zebrafish and *Caenorhabditis elegans* were used for the evaluation of efficacy of drug molecules. However, the majority of experiments were conducted using transgenic (Tg) mice (Langley, 2014). Apart from the above mentioned transgenic mice models, aluminium chloride-induced Alzheimer's disease rat models are also used in the preclinical studies of Alzheimer's disease drugs. Aluminium chloride induces pathological changes such as oxidative stress and neurodegeneration, which were similar to Alzheimer's disease pathology (Boegman & Bates, 1984; Nehru & Anand, 2005, Ramachandran *et al.,* 2013). In addition, aluminium has been suggested as one of the risk factor in the pathogenesis of early stage of Alzheimer's disease (Rebai *et al.,* 2008; Kumar *et al.,* 2011). Mainly, aluminium chloride-induced neurotoxic effects are oxidative damage due to increased free radicals synthesis (Boegman & Bates, 1984; Donald., 1989; Lebel & Bondy, 1991; Yokel, 2001). Besides, it was also found to suppress the anti-oxidant enzymes (Superoxide dismutase and catalase) and causes increased lipid peroxidation (Nehru &

Anand, 2005). Other common Alzheimer's disease like features are increased levels of Aβ protein, development of hyperphosphorylated tau protein, degeneration of cholinergic terminals in cortex and hippocampus, and finally, neuronal apoptosis (Khan *et al.,* 2013). Aluminium is known to get accumulated in the hippocampal region of the brain, subsequently affecting the learning and memory (Lal *et al*., 1993; Julka *et al*., 1996). It was also shown that, the total PP2A activity goes down upon treatment with aluminium chloride (Walton, 2012). One of the main reasons for tau hyperphosphorylation seems to be the imbalanced activity of protein kinases in the brain. Protein Phosphatase (PP2A) activity goes down during the pathophysiology of Alzheimer's disease leading to abnormal hyperphosphorylation of tau (Iqbal *et al*., 2005). Aluminium was shown to inhibit protein phosphatase which is correlated with hyperphosphorylation of tau, leading to the accumulation of NFTs in the brain (Walton, 2012). Enhancing the activity of PP2A may alleviate the hyperphosphorylation of tau and prevent subsequent accumulation of NFTs in the brain.

Based on the available literature, aluminium induced rat model of Alzheimer's disease was used in the present research work for the evaluation of neuroprotective efficacy of BMC. Rats of both the sexes (Male and female) were used in order to avoid the gender based differences. Since Curcumin was already studied by other researchers for the treatment of Alzheimer's disease, only BMC was considered for the animal model study. The neuroprotective efficacy of BMC in aluminium induced Alzheimer's disease model was evaluated in male and female Sprague-Dawley rat after an oral dose of 500 mg/kg body weight per day. In this animal model, various parameters have been investigated which includes, estimation of lipid peroxidation in brain, superoxide dismutase activity in brain, circulating superoxide dismutase activity in blood, protein phosphatase 2A (PP2A) activity as well as histopathology of the brain.

BMC, at tested dose showed potent neuroprotective activity as evidenced from the results. Lipid peroxidation was high in the disease group implicating increased oxidative stress upon administration of aluminum chloride. These findings were similar to those previously reported by Aly *et al.*(2011) using the similar dose of aluminum chloride.

Oral administration of BMC, significantly ($P<0.05$) reduced the Lipid Peroxidation (LPO) when compared to the disease control (3.5±0.14 nmol/g and 8.8±0.20 nmol/g for BMC and disease group, respectively). Lipid peroxidation in BMC group was comparable to control group (3.5±0.14 nmol/g and 3.0±0.16 nmol/g for BMC and control group, respectively), showing the antioxidant activity of BMC in *in vivo* conditions.

Also, BMC significantly ($P<0.05$) increased the levels of SOD activity as compared to disease group in the brain (822.2±15.90 units/g and 433.4±17.71 units/g for BMC and disease group, respectively), plasma (434.8±13.30 units/g and 245.1±17.22 units/g for BMC and disease group, respectively) and in RBC lysate (852.4±23.54 units/g and 404.4±7.28 units/g for BMC and disease group, respectively). No significant difference was observed for SOD activity between BMC group and control group in the brain (822.2±15.90 units/g and 807.7±16.26 units/g for BMC and control group, respectively), plasma (434.8±13.30 units/g and 435.5±13.62 units/g for BMC and control group, respectively) and RBC lysate (852.4±23.54 units/g and 871.0±19.32 units/g for BMC and control group, respectively). Oral administration of BMC at the dose of 500mg / kg body weight was found to combat oxidative stress by enhancing the activity of SOD in aluminium chloride-induced animal model of Alzheimer's disease. These findings are in line with the previously reported results from *in vitro* studies, where BMC was proven to be a potent antioxidant (Abas *et al.,* 2006; Ravindran *et al.,* 2010).

BMC was also found to enhance the activity of PP2A enzyme in aluminium chloride-induced rat model of Alzheimer's disease. PP2A is a phosphatase enzyme found in most of the tissues. It is an important tau dephosphrylation enzyme in the brain and its lower activity is linked to hyper phosphorylation of tau proteins on microtubules, which leads to destabilization of microtubule (Torrent *et al.,* 2012). Significant decrease in total PP2A activity was observed in cortical and hippocampal regions in the Alzheimer's disease brain. This was correlated with the tau pathology due to hyperphosphorylated tau protein in Alzheimer's disease brain (Walton, 2007). Additionally, lower enzymatic activity of PP2A is linked to neuronal cell death in the brain, probably due to destabilization of microtubules and accumulation of NFTs. Inhibitors of phosphatase enzymes were shown to induce cognitive decline, accumulation of phosphorylated tau, accumulation of amyloid peptides

and subsequently, neurodegeneration. Aluminium was shown to induce hyperphosphorylation of tau via inhibition of PP2A activity, leading to formation and accumulation of NFTs (Walton, 2007; 2012). Compounds such as Sodium selenate was found to increase PP2A activity, subsequently, reversing tau phosphorylation (Sontag *et al.,* 2014). Enhancing the PP2A activity via therapeutic interventions in brain would decrease tau hyper-phosphorylation that polymerizes to form NFTs (Walton, 2007). Hence, it is considered as a good drug target in the therapeutic intervention for Alzheimer's disease (Torrent *et al.,* 2012). The PP2A enzyme activators can be a potential drug molecule in the therapeutic intervention for Alzheimer's disease.

In the aluminium chloride-induced rat model, PP2A activity was significantly ($P<0.05$) decreased in disease group (0.8±0.07 pmol/µg of protein) when compared to control group(3.7±0.17 pmol/µg of protein). These data reconfirm the previously reported research work on aluminium induced Alzheimer's disease animal model (Walton, 2007; 2012). In BMC group, PP2A activity was significantly ($P<0.05$) increased when compared to disease group (3.4±0.14 and 0.8±0.07 pmol/µg of protein for BMC group and disease group, respectively). PP2A activity of the BMC group was comparable to control group. Increased PP2A activity in BMC group might lead to the reduced hyperphosphorylation of tau and consequently, reduced neurofibrillary tangles (Walton, 2007) thus, decreasing neurological damage in Alzheimer's disease. In the present study, BMC was found to be an effective neuroprotective compound in aluminium chloride-induced Alzheimer's disease rat model.

8.2 Conclusion:

Multiple pathways and risk factors are suggested in the pathophysiology of Alzheimer's disease, which led to the identification of several drug targets in the development of therapeutic interventions. It is unlikely that, the drug acting on a single target will be helpful in the treatment of Alzheimer's disease (Russo *et al.,* 2013). Thus necessitating the need for multi-target drug in the effective treatment of Alzheimer's disease. Multi-target drugs are often phytochemicals (Russo *et al.,* 2013), which are known for higher safety profile.

In the present research work, BMC showed multi-target drug potential with antioxidant, anti-inflammatory, BACE1 inhibition and enhancing PP2A enzyme activity. It was also found to be safe at the oral dose of 500 mg/kg body weight in rats. The results from present research work showed that, BMC is a potential drug candidate in the therapeutic intervention for Alzheimer's disease. Also, the present research work established a good correlation between *in silico*, *in vitro* and *in vivo* studies. Further studies are required to be carried out in transgenic mice to evaluate the efficacy of BMC in the treatment of Alzheimer's disease.

Chapter-9

9. Future research:

In the present research work, BMC was found to be a potential multi-target drug candidate with antioxidant, anti-inflammatory, BACE1 inhibition and PP2A activity enhancing property. It was also proven to be superior antioxidant and anti-inflammatory agent compared to Curcumin, which is already proven to be safe and effective in the treatment of Alzheimer's disease. Although, BMC has showed good activity in aluminum chloride-induced Alzheimer's disease model, its efficacy should to be further studied in specific animal model for BACE1 inhibition and PP2A enhancing activity. The pharmacological properties of BMC have to be explored and studied further. Subsequently, ED_{50} has to be evaluated in the transgenic mice model for specific drug target. BMC has a poor aqueous solubility and hence development of a robust and highly bioavailable formulation is necessary before conducting any *in vivo* studies. Once the mechanism of action and ED_{50} are thoroughly studied, it can be further taken through safety studies and efficacy studies as per the regulatory requirement to market the drug in respective countries.

The steps for further research to develop BMC as a drug for the therapeutic intervention in Alzheimer's disease are listed below.

1. Evaluation of physicochemical properties of BMC
2. Development of a robust and bioavailable formulation
3. *In vitro* efficacy studies
4. Pharmacokinetic studies in animals
5. Efficacy studies in *in vivo* animal models; Transgenic mice
6. Safety studies as per the regulatory guidelines for a new drug
7. Clinical studies as per the regulatory guidelines for a new drug

Chapter-10

10. References:

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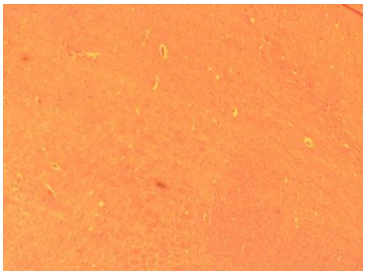
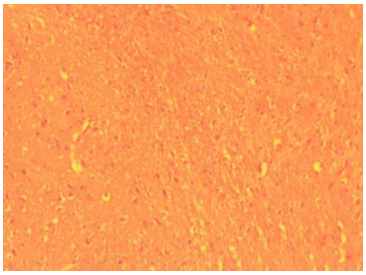
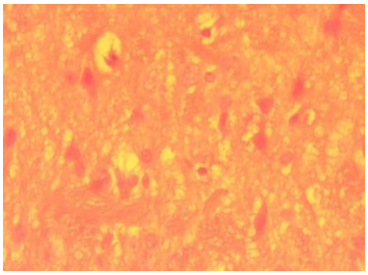
Chapter-11

11. List of Appendices:

11.1 Appendix A: Histopathology brain samples:

a. Control group : **Male rat-1**

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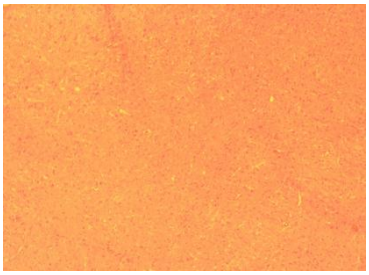
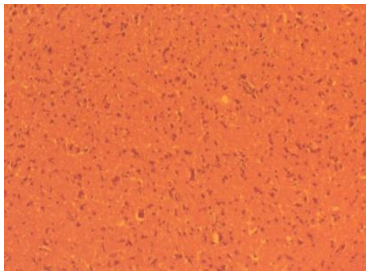
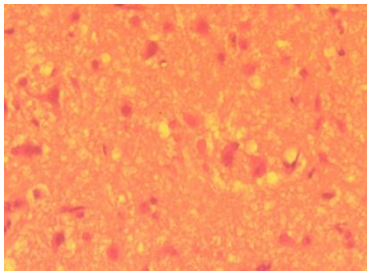
Male rat-2

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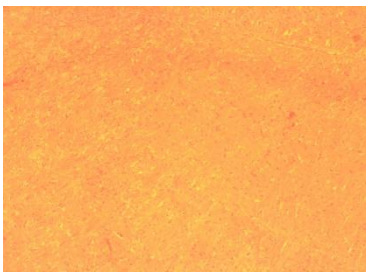
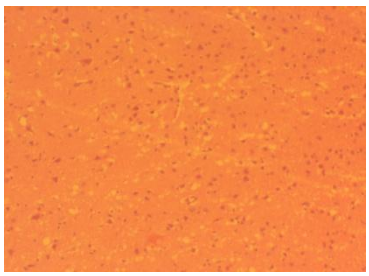
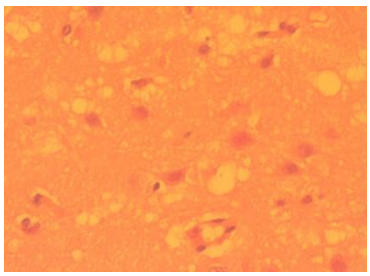
Male rat-3

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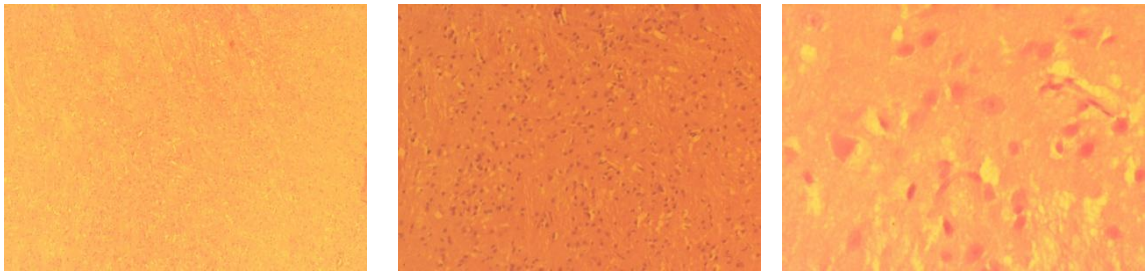
Male rat -4



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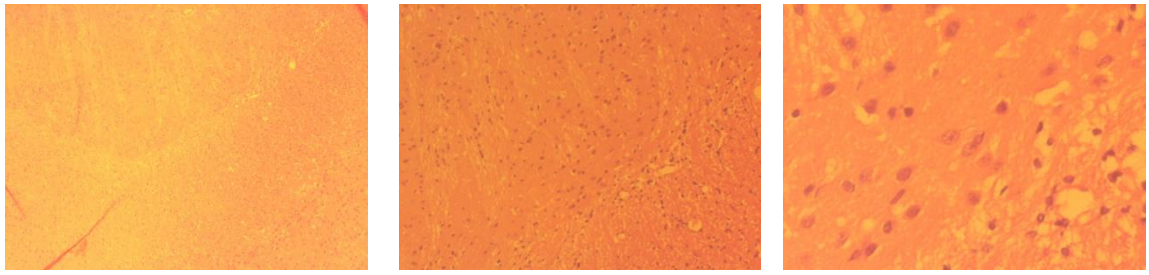

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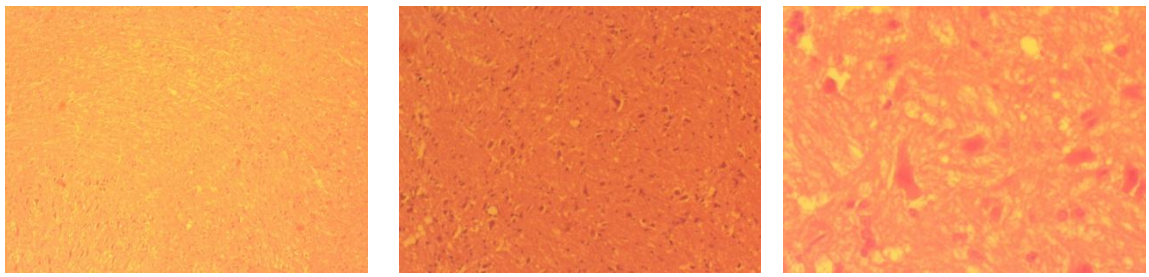
Male rat -6



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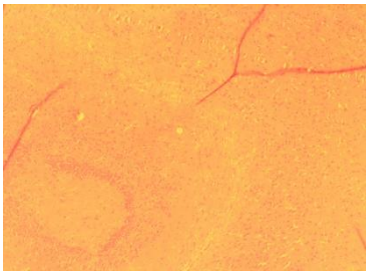
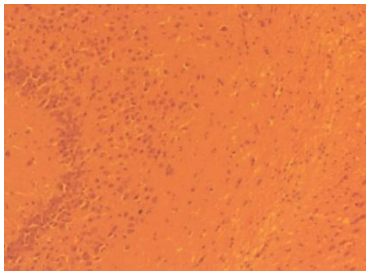
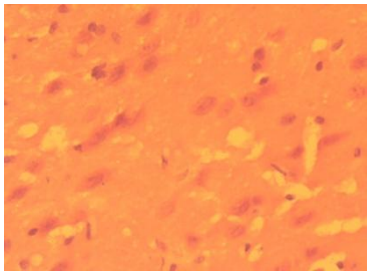
g. Disease group :

Male rat -1

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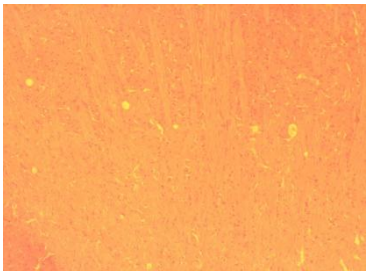
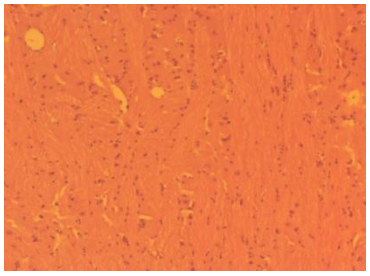
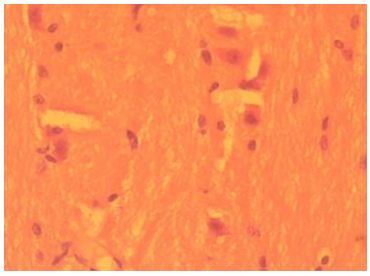
Male rat -2

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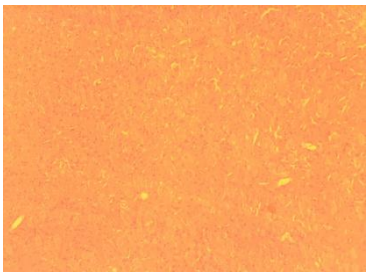
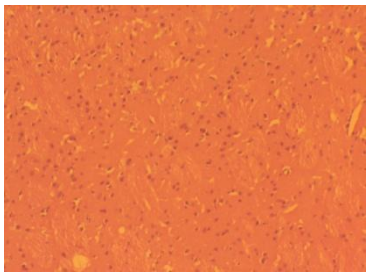
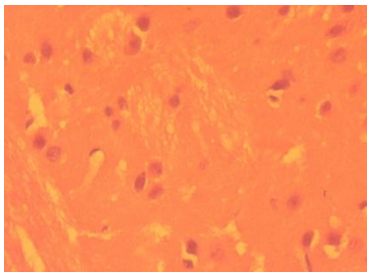
Male rat -3

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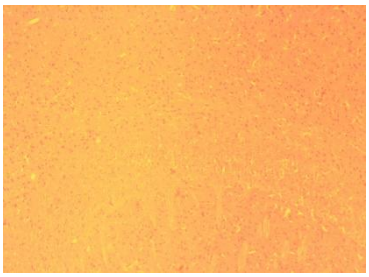

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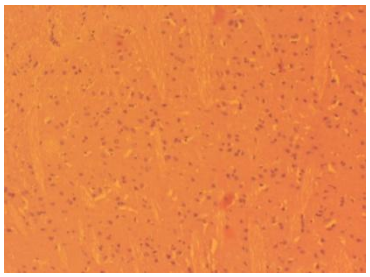

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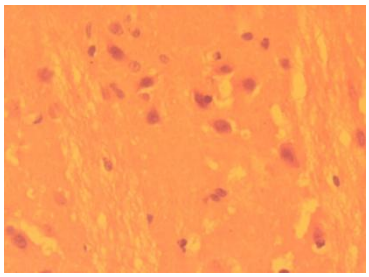
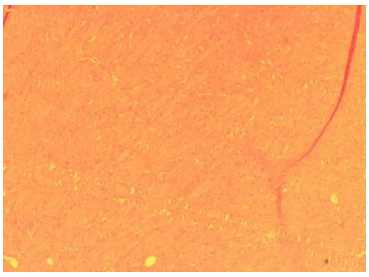


(40X)

Male rat -5



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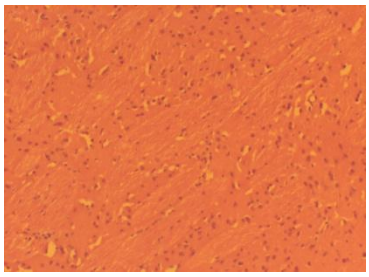
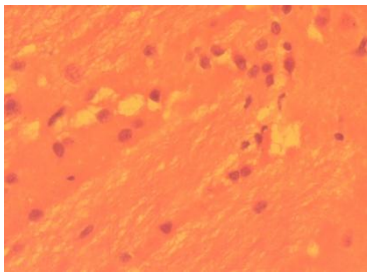
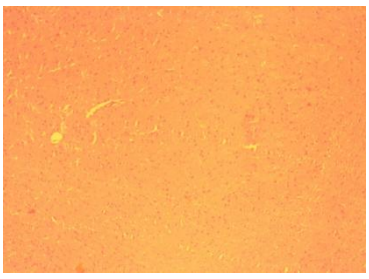


(10X)



(40X)

Male rat -6



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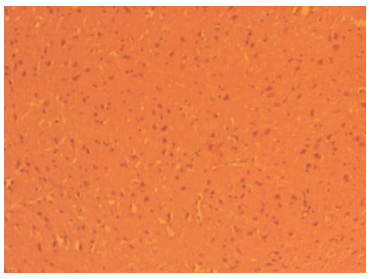

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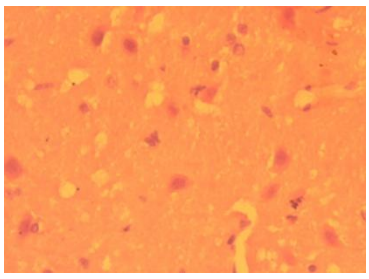
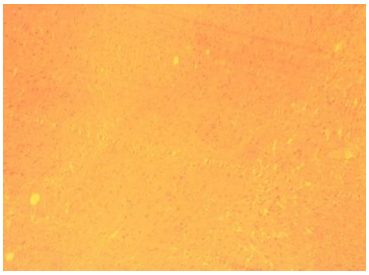
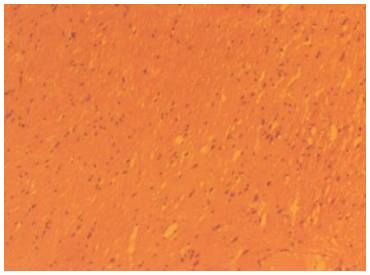
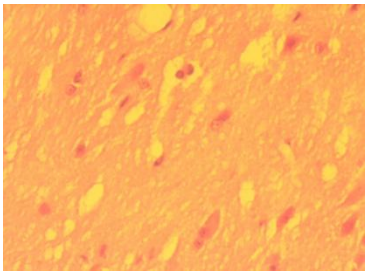


(40X)

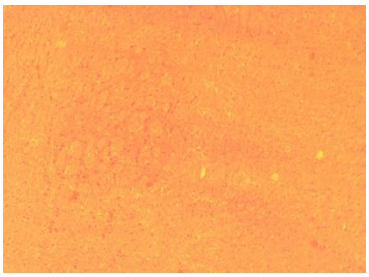
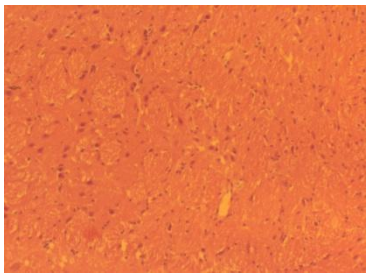
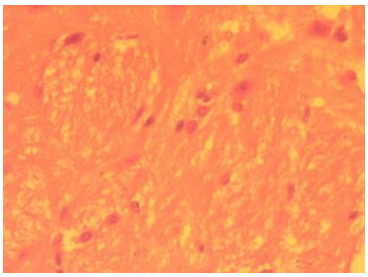
h. Test group :

Male rat -1

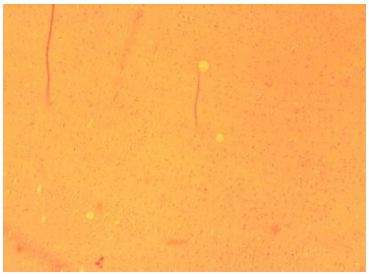
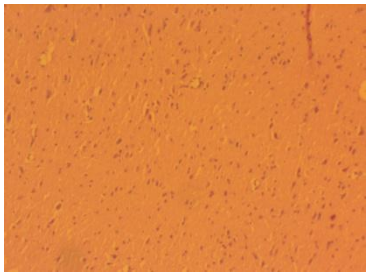
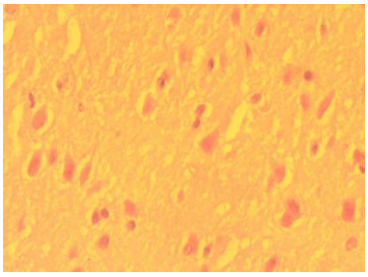
(4X) **(10X)** **(40X)**

Male rat -2

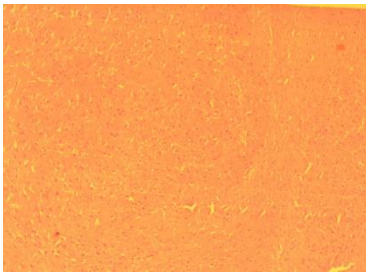
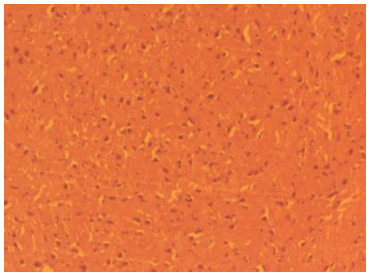
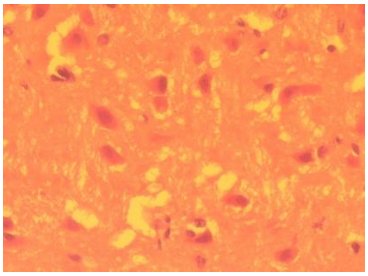
(4X) **(10X)** **(40X)**

Male rat -3

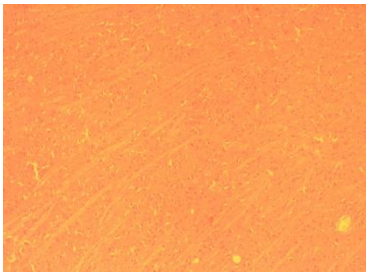
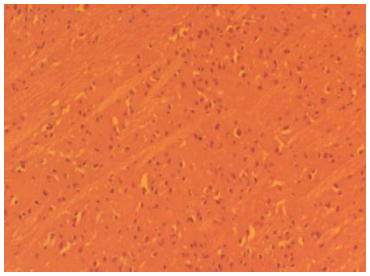
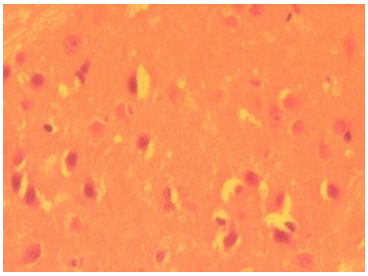
(4X) **(10X)** **(40X)**

Male rat -4

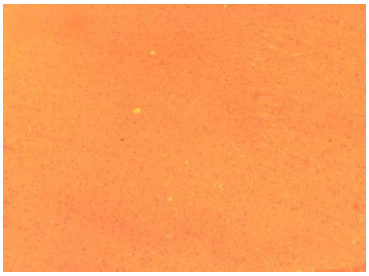
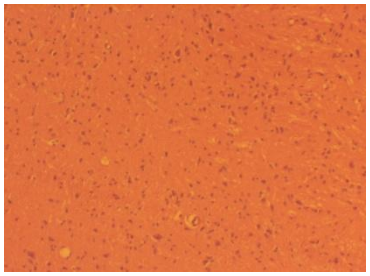
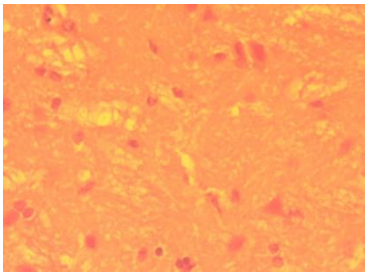
(4X) **(10X)** **(40X)**

Male rat -5

(4X) **(10X)** **(40X)**

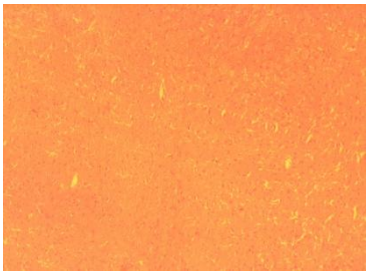
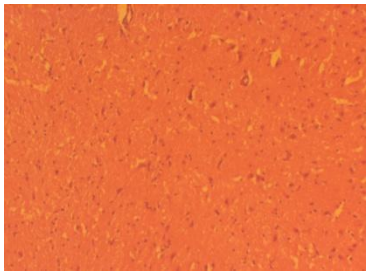
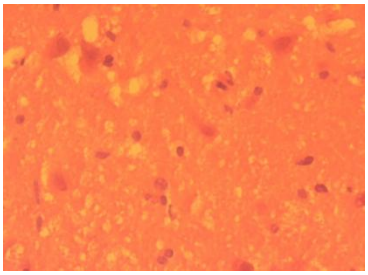
Male rat -6

(4X) **(10X)** **(40X)**

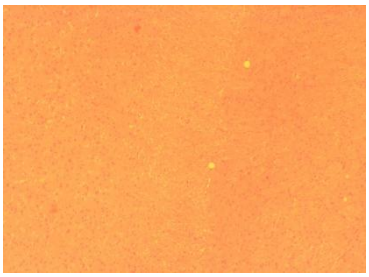
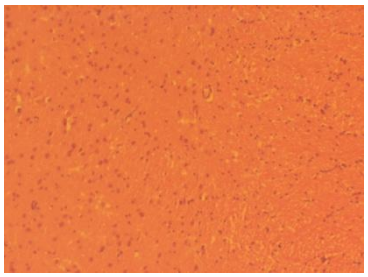
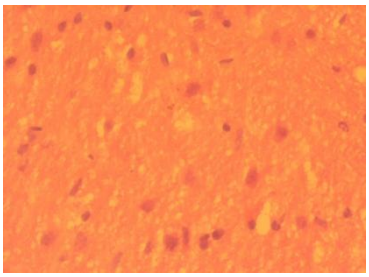
i. **Control group :**

Female rat -1

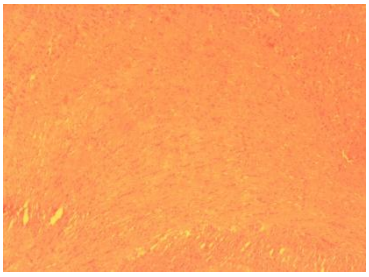
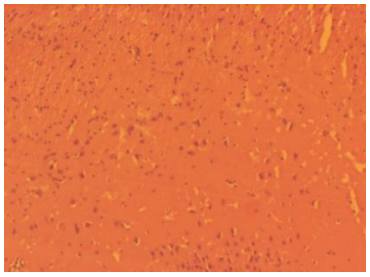
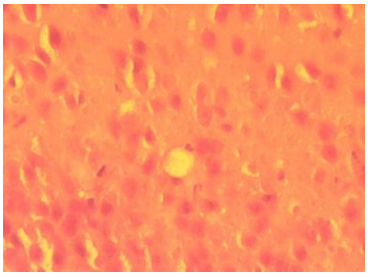
(4X) **(10X)** **(40X)**

Female rat -2

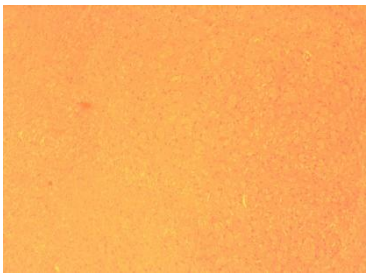
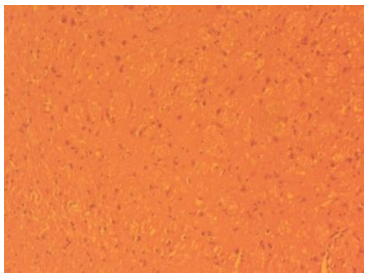
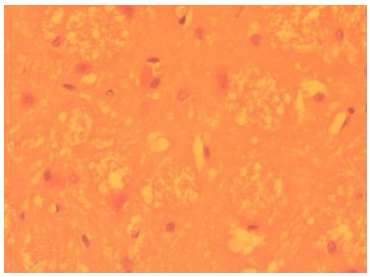
(4X) **(10X)** **(40X)**

Female rat -3

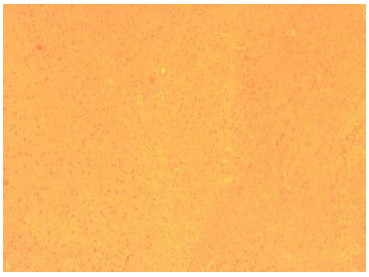
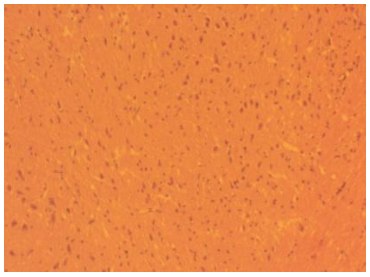
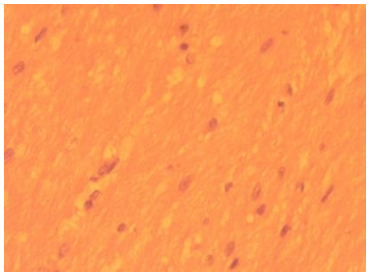
(4X) **(10X)** **(40X)**

Female rat -4

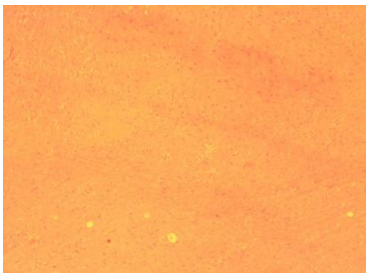
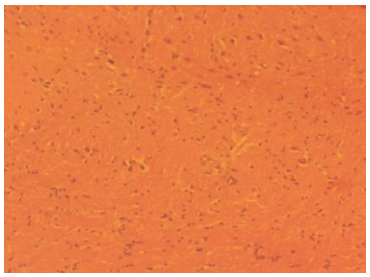
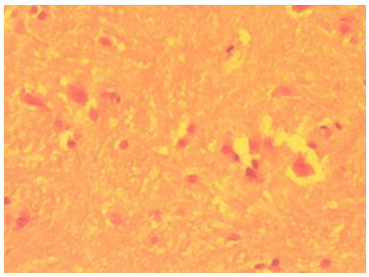
(4X) **(10X)** **(40X)**

Female rat -5

(4X) **(10X)** **(40X)**

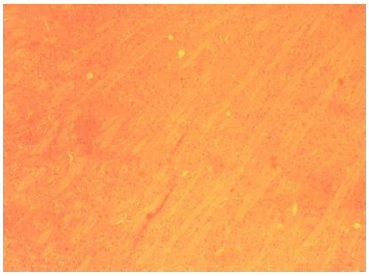
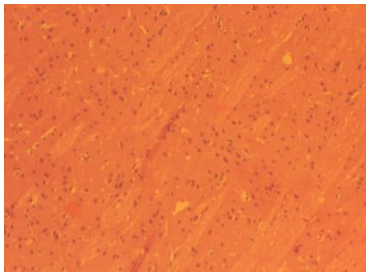
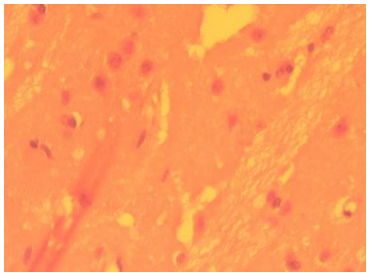
Female rat -6

(4X) **(10X)** **(40X)**

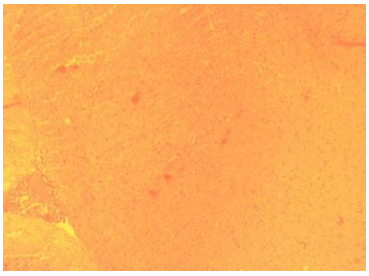
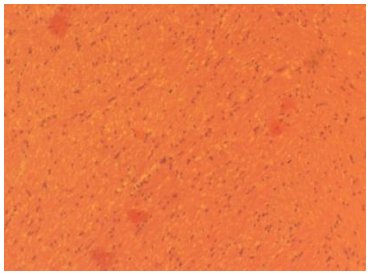
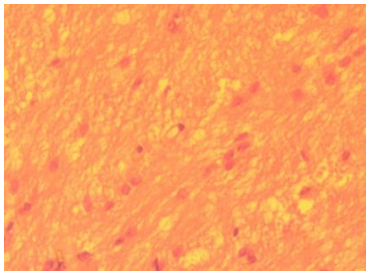
j. Disease group :

Female rat -1

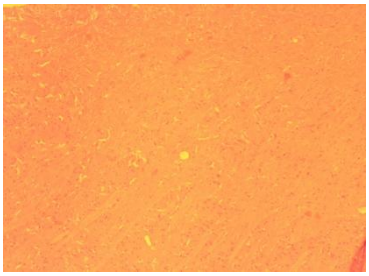
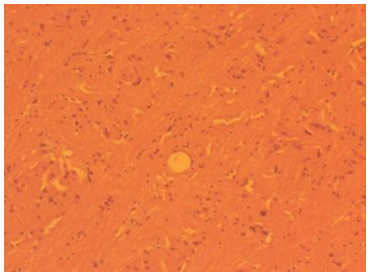
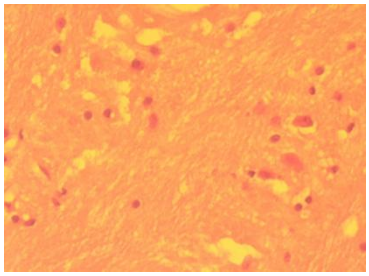
(4X) **(10X)** **(40X)**

Female rat -2

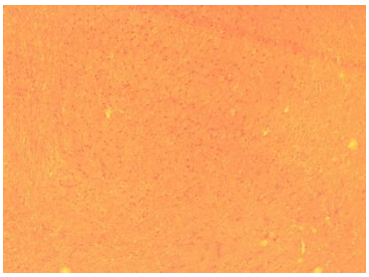
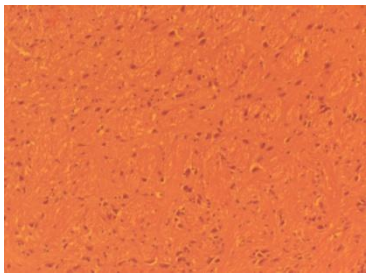
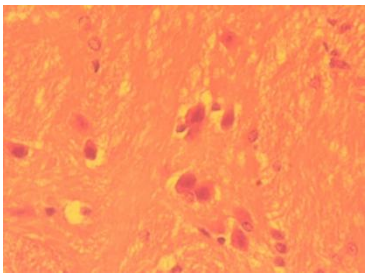
(4X) **(10X)** **(40X)**

Female rat -3

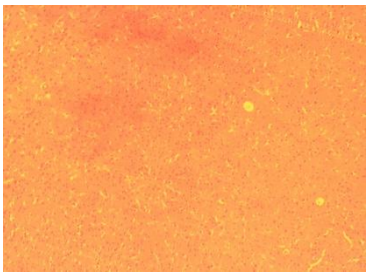
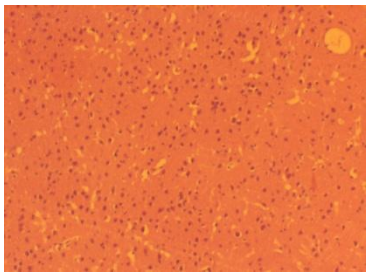
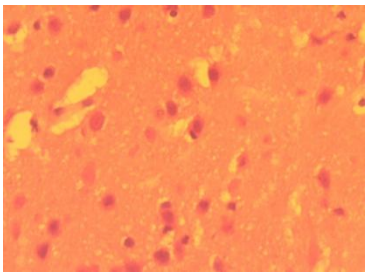
(4X) **(10X)** **(40X)**

Female rat -4

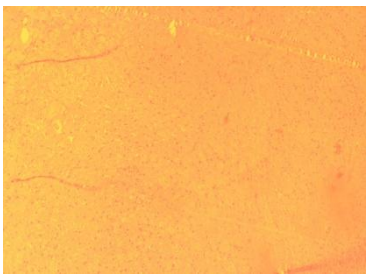
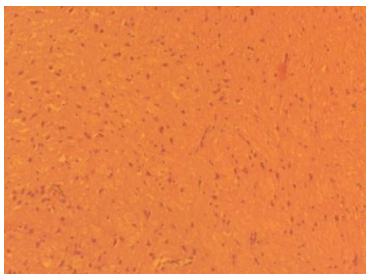
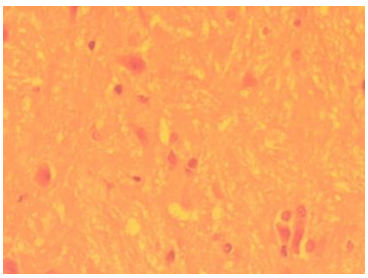
(4X) **(10X)** **(40X)**

Female rat -5

(4X) **(10X)** **(40X)**

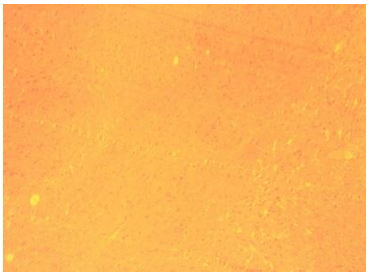
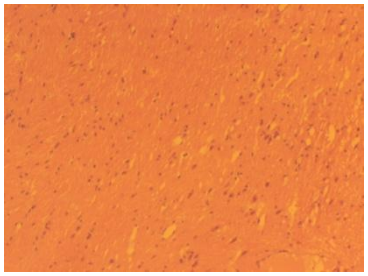
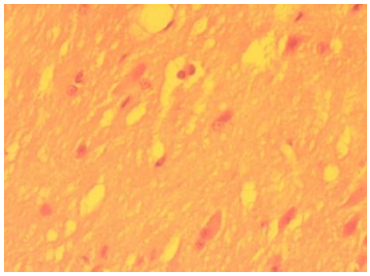
Female rat -5

(4X) **(10X)** **(40X)**

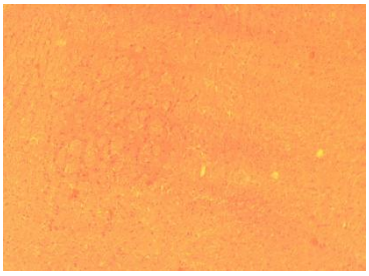
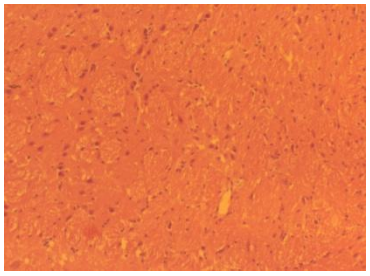
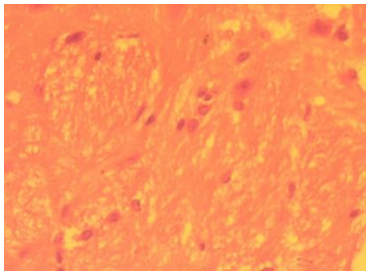
k. Test group :

Female rat -1

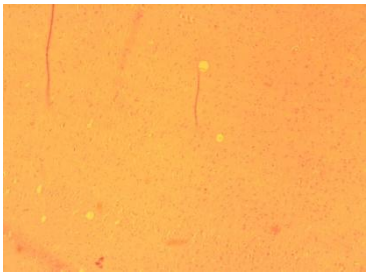
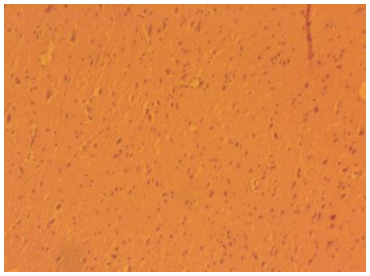
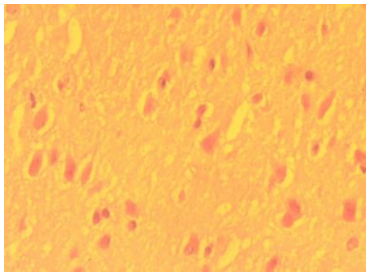
(4X) **(10X)** **(40X)**

Female rat -2

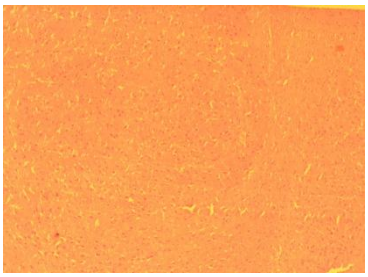
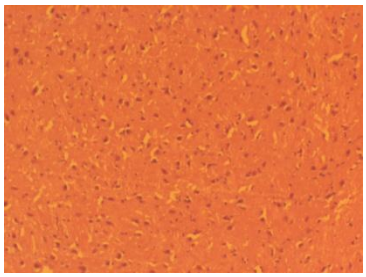
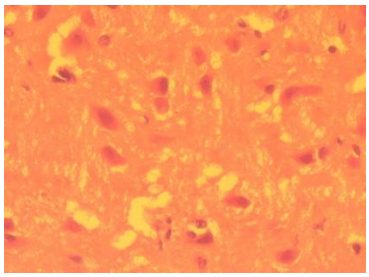
(4X) **(10X)** **(40X)**

Female rat -3

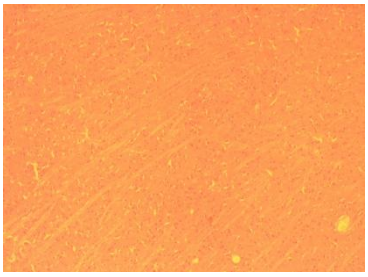
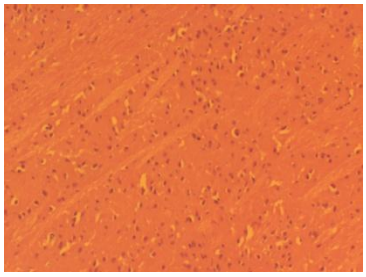
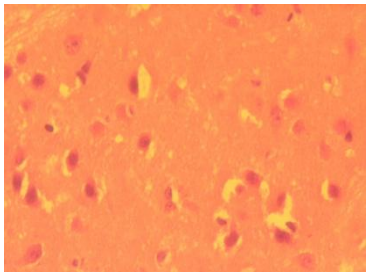
(4X) **(10X)** **(40X)**

Female rat -4

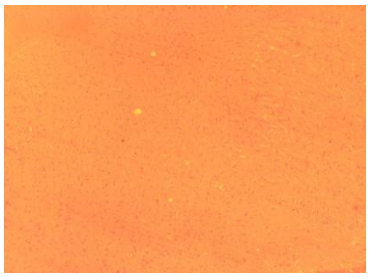
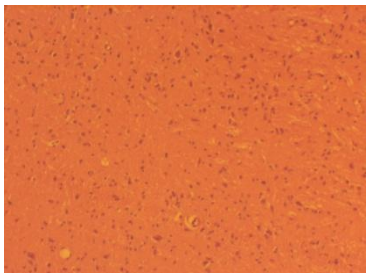
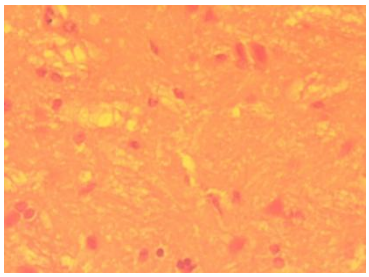
(4X) **(10X)** **(40X)**

Female rat -5

(4X) **(10X)** **(40X)**

Female rat -6

(4X) **(10X)** **(40X)**