CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Screening of Active Components in Ajuga *Bracteosa* Effective Against Alzheimer's Disease

by

Aleena Ijaz

A thesis submitted in partial fulfillment for the degree of Master of Science

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I dedicate this thesis to my loving family who prayed for me and supported me every step of the way

CERTIFICATE OF APPROVAL

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Aleena Ijaz

(MBS203002)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Faiza Munir	ASAB NUST, Islamabad
(b)	Internal Examiner	Dr. Sohail Ahmad Jan	CUST, Islamabad
(c)	Supervisor	Dr. Erum Dilshad	CUST, Islamabad

Dr. Erum Dilshad
Thesis Supervisor
October, 2022

Dr. Syeda Marriam Bakhtiar
Head
Dept. of Biosciences & Bioinformatics
October, 2022

Dr. Sahar Fazal
Dean
Faculty of Health & Life Sciences
October, 2022

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Abstract

Alzheimer is a neurodegenerative disease and a common form of dementia that occurs as a consequence of aging. Advancements in screening techniques and treatment methodologies have proven significant role in reducing the symptoms however adverse effects are still considerable. The detailed study of the disease showed that hydrolase enzyme, Acetylcholinestrase and Butyrylcholinestrase present in synaptic cleft of the brain hydrolyze the acetylcholine into acetate and choline that causes termination of neuron signals. This results in cholinergic deficit and shrinking of brain tissue. Globally people are more inclined in using plant based products than synthetic ones. That's why this research is planned to determine cholinesterase inhibitors present in *Ajuga bracteosea* for the treatment of Alzheimer disease. Twenty one bioactive compounds were selected from the plant for this purpose. The structure and physiochemical properties of these ligands were studied. The ligands were virtually screened against drug targets that are AChE and BChE. Ligands and proteins were docked using CB dock and visualized through PyMol and analyzed through LigPlot. These ligands were then screened out based on Lipinski rule and their ADMET properties were studied. Ellagic acid was selected as leading compound against, Acetylcholinestrase and Butyrylcholinestrase receptors. The comparative results of selected lead compound with standard drug, donepezil showed less toxicity and far more activity. However, further research has to be carried out to investigate its potential medicinal use.

Keywords: Dementia, Acetylcholinestrase, Butyrylcholinestrase, *Ajuga bracteosea*, CB dock, PyMol, ADMET, Ellagic acid, donepezil

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Abbreviations

A.bracteosa	*Ajuga bracteosa*
AD	Alzheimer disease
ACh	Acetylcholine
ACHE	Acetylcholinestrase
APP	Amyloid β precursor protein
BCHE	Butyrylcholinestrase
BBB	Blood Brain Barrier
CNS	Central Nervous System
FDA	Food Drug Authority
MMSE	Mini Mental State Examination
MRTD	Maximum rate tolerated dose
PDB	Protein Data Bank
VDss	Volume of Distribution

Chapter 1

Introduction

1.1 Background

Dementia is a general term for a group of diseases affecting humans that damage the ability of a person to think, remember, and make decisions hence affecting an individual's day to day life. Alzheimer is a neurodegenerative disease that is a common form of dementia. It is a rare disorder that was considered to occur as a consequence of aging as it mostly affects the people in the age group of 60s or above. In 1906 a clinical psychiatrist and neuroanatomist, Dr. Alois Alzheimer discovered this disease while investigating the brain of a 50 year old women who died of an unknown mental illness [1]. She showed the symptoms of paranoia, aggressiveness and speech and memory loss before death.

Today, this disease has a global pervasiveness of 3.9% in people aged 60 above with the zonal spread of 19.4 per 1000 person in Europe, 3.7 in Brazil, 3.2 in India and 1.39 in Pakistan [2]. Various composite pathogenic mechanisms cause the progression and onset of Alzheimer disease and these include defects in cholinergic function, disruption of blood brain barrier, oxidative stress, inflammation and formation of plaques and tangles. The damage initially occurs in the hippocampus of the brain, as the disease progresses more neurons continue to die. At the final stage the brain tissue has shrunk significantly. Symptoms associated with

this disease include cognitive disruption, memory loss, language disturbance, personality changes, sleep deprivation and reduce judgment abilities. The symptoms of AD slowly become more severe with a loss of 3 to 4 points on the instrument such as Mini Mental State Examination. (MMSE) [3]. In the early years patients usually suffer from memory loss followed by loss of sensory and motor function. Late stages of the disease lead to becoming mute and bedridden. After the diagnosis the average life span of the patient is 8 to 10 years, however the disease can last up to 20 years [4]. Alzheimer's disease progression is caused by the cholinergic transmission deficits with the reduction of neurons. The acetylcholinestrase (AChE) and Butyrylcholinestrase (BChE) are the two main neurotransmitters of 531 and 529 residues that are present in the synapse between nerve and muscle cells. These hydrolytic enzymes hydrolyze the acetylcholine (ACh) that is released in the synaptic cleft into acetate and choline for termination of the neuron signals [5]. With this termination the pieces are taken back to rebuild new neurotransmitters. As the diseaseprogresses there is a decrease in nerve cells which cause the deficiency of acetylcholine in the brain. Thus due to the major role played by AChE and BChE they are selected as potential target for the development of drug that can inhibit their action and used against Alzheimer's [6].

To this date FDA has approved many drugs that have shown promising outcomes and have reduced the progress of disorder, however public health information is limited when it comes to exact causes of Alzheimer. The process of Alzheimer disease has a direct relationship with the behavioral and pathological changes in the brain of individuals so medications can be developed that can halt the disease [7]. Most information gathered during the drug discovery indicated that the breakdown of acetylcholine worsens the disease so a number of drugs were produced to improve the cholinergic symptoms hence increasing the life span of patients but the death of neurons still seems like an inevitable scenario.

Pharmacological choices available for Alzheimer treat cognitive defects and mood stabilizers and antidepressants are available for treatment of behavioral changes. Acetylcholinestrase inhibiters are the only agents approved by FDA for the treatment of the disease. These drugs have shown positive results during the middle

stages of the disease [8]. These drugs have limited efficiency as they produce adverse reactions such as fever, nausea, dizziness, diarrhea and anorexia [9].

Taking into account the new strategies for the development of drugs against the Alzheimer disease several natural compounds were identified by phytochemical studies. These compounds have previously shown to possess diverse biological and pharmacological activities. AChE and BChE being the potential targets for inhibitor drugs have shown strong binding affinity with these bioactive compounds. Unsurprisingly, appreciable efforts have been made to develop anti Alzheimer drugs from these natural compounds. The process of drug development is both time and effort consuming yet there is dire need of medicines for treatment of numerous diseases. Therefore to facilitate this process in-silico virtual screening and computer based methods such as molecular docking have been in use from past few decades.

Virtual screening is a low cost and direct drug discovery approach as compared to experiment methods. This can be done by ligand based and structure based methods. Molecular docking is a structural based tool. It is a computational method designed to study the binding of molecules with the target proteins through specialized scoring function and predicting the structures at atomic level. The software used for molecular docking are AutoDock4, Auto Dock Vina, CB Dock, Flexx, Glide and ICM. Molecular docking provides an economic and a quick method for high throughput screening for drug design and development [10].

The use of medicinal plants and herbs to treat dementia and other disorders date back to a long time ago. Natural compounds produce from herbs not only have a high therapeutic value but are also used to produce new compounds against diseases with more efficiency and reduced side effects [11].

Herbs have anti-inflammatory and antioxidant properties that can be beneficial for the treatment of Alzheimer disease. Therefore attempts have been made to identify bioactive compounds from these plants that can cause the inhibition of these hydrolase enzymes and used against the disease. AChE and BChE have been proved to be target enzymes to treat the cholinergic deficit and are the target sites used for screening the active compounds of medicinal plants.

1.2 Problem Statement

Alzheimer disease is the most common cause of dementia around the world effecting 1 to 4 percent of people of age 65+ and 47 percent of people over the age of 85 in population every year.

It is estimated that these statistics will triple in next 30 to 40 years [12]. It is recognized as a global threat by WHO because of its high mortality rate and lack of known causes and treatment.

This study covers the pharmacological activities of compounds derived from *Ajuga bracteosa* and their therapeutic application in Alzheimer disease by targeting the hydrolase enzymes Acetylcholinestrase (AChE) and Butyrylcholinestrase (BChE) for the conduction of in-silico studies through molecular docking.

1.3 Aims and Objectives

The main aim of this study is to identify cholinesterase potential inhibitors against hydrolase enzymes (AChE and BChE) by using computational tools such as molecular docking of bioactive compounds of *Ajuga bracteosa* that show strong affinity with AChE and BChE to help design drugs for treatment of Alzheimers.

The objectives of this study are:

- To identify bioactive compounds and their derivatives in *Ajuga bracteosa* as potential inhibitors of AChE and BChE.
- To study the interaction between AChE and BChE as targeted proteins and active compounds in *Ajuga bracteosa* as ligands through molecular docking.
- To compare the results of inhibitor and ligands and finding the best interacting molecules that show inhibitory effects against the disease.

1.4 Scope

So far there is inadequacy in interpretation of the exact pathophysiology of Alzheimer disease and is a great challenge in developing drugs against this devastating disease. A major pitfall in developing drugs is the unavailability of accurate data, for this reason the efficiency of drugs is limited. Natural products are considered to hold enormous potential in therapeutics. Several natural compounds are found to be effective against Alzheimer disease. So there is a need for discovery of other plant based products that have inhibitory properties against the cholinesterase enzymes. In-silico molecular docking can help in the identification of such compounds against AChE and BChE that could prove beneficial in the treatment of Alzheimer disease.

Chapter 2

Literature Review

2.1 Alzheimer's Disease

Alzheimer disease is one of the most destructive disorders of elderly humans. It is a rare condition that is under recognized but is becoming a major health problem since the last decade. Alzheimer's is a gradually increasing neurologic disorder characterized by cognitive defects and behavioral disturbances. Cognitive defects and neurological conditions occur because of the aggregation of oxidative damage to nucleic acid, mitochondria and proteins of the brain [13]. It causes the

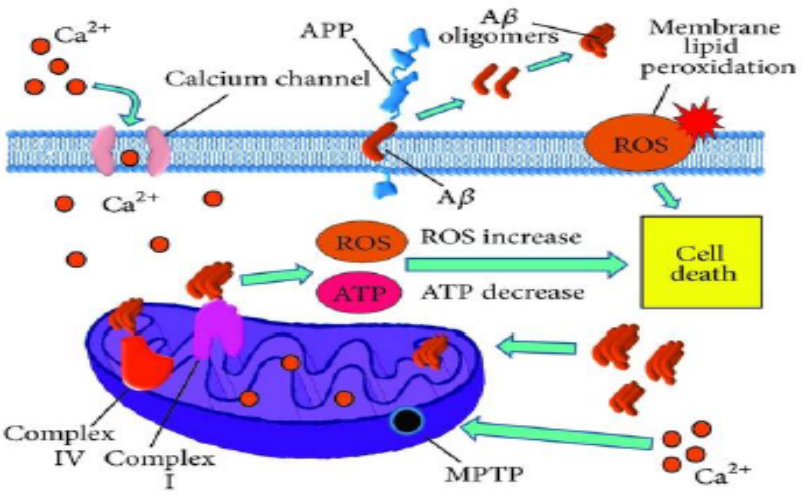


FIGURE 2.1: Cognitive defects and cell death due to oxidative damage [13].

atrophy of the brain cells eventually resulting in the death of the cells. The symptoms continue to become worse over time and the disease is lethal. In 1906 Dr. Alois Alzheimer discovered this disease after noticing certain abnormal clumps and tangles caused by an unknown mental illness in the brain of a dead patient. Alzheimer's disease has not only affected the elderly but is also becoming a threat for people below the age of 65. It plays havoc with the normal day to day life of individuals and creates a burden both socially and economically [14].

2.2 Signs and Symptoms of Alzheimer's Disease

The signs and symptoms of Alzheimer's disease range from mild to severe. Patients suffering from Alzheimer's show memory impairment in the early stages of the disease. It affects an individual's thinking and concentration abilities making it difficult to make decisions that affecting the tasks of daily life. In the late stages loss of sensory and motor functions, language disturbances are usually shown by the patients [15]. Recent studies indicate that almost 85% of the individuals also have non cognitive symptoms such as changes in personality and behavior, mood swings, weight loss, sleep abnormalities, seizures, loss of bowel and bladder control and skin infections. Patients eventually become silent and confined to bed. Once diagnosed with the disease patients on average usually live 8 to 10 years.

2.3 Causes of Alzheimer's Disease

In the past decade several causes of the Alzheimer's disease have been identifies. These include;

2.3.1 The Amyloid Cascade Hypothesis

Formation of two abnormal structures, Amyloid plaques and neurofibrillary tangles in the brain of patients is considered as one of the major cause of Alzheimer's

disease. These plaques are formed in hippocampus of the brain. Plaques are insoluble deposits of a protein known as Amyloid Precursor Protein (APP). Amyloid precursor protein has 695 to 770 amino acid long structure and its gene is present on chromosome 21 [16].

This protein is sequentially cleaved by 2 main enzymes known as β secretase and γ secretase. Other molecules such as Amyloid β (Aβ) and sAPPβ are also produced which may also play a role in the progression of the disease. A β misfolds on itself and sticks with other molecules to form oligomers. These oligomers are insoluble which aggregate in the brain as senile plaques.

Figure 2.2 highlights the processes involved in the formation of plaques. These plaques weaken the communication at synapses that then stops the brain from restoring memories. Apart from plaque formation Aβ also forms neurofibrillary tangles that cause the death of neurons.

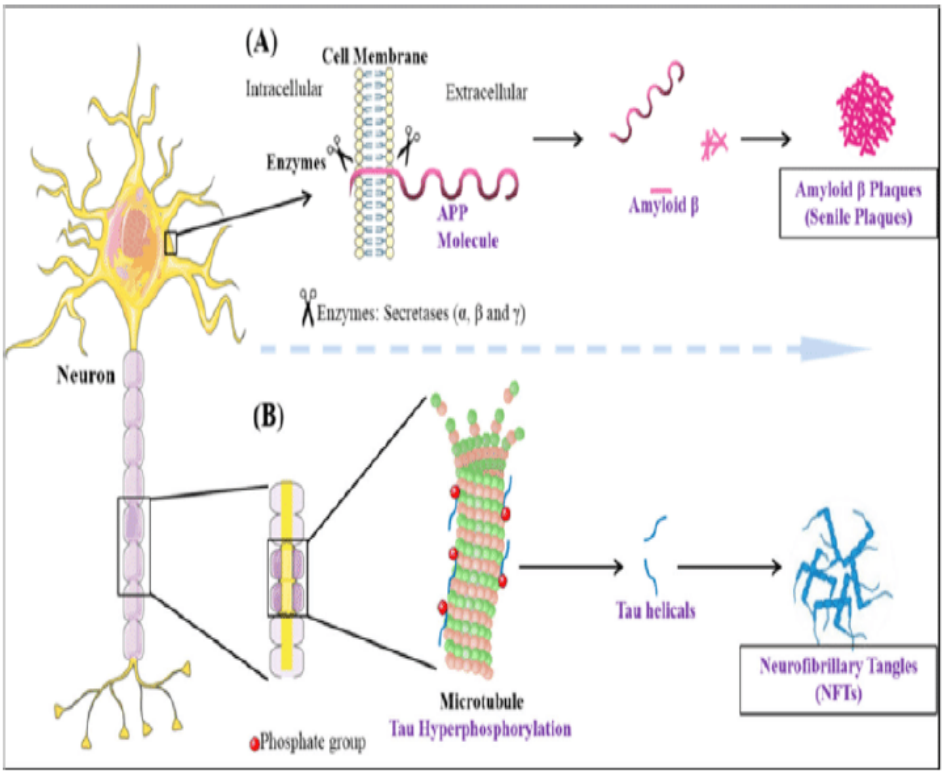


FIGURE 2.2: The neuropathological cause of Alzheimer's disease: (A) Amyloid-beta plaques formation (B) Formation of neurofibrillary tangles [16].

2.3.2 Cholinergic Hypothesis

Another possible cause of Alzheimer disease is cholinergic deficit. Cholinesterase enzymes exist in two forms Acetylcholinestrase (AChE) and Butyrylcholinestrase (BChE). These enzymes are involved in the cleavage of acetylcholine (ACh) in the synaptic cell. The process of cholinergic synapse is summarized in **Figure 2.3**. In disease conditions there is low level of ACh but the activity of cholinesterase enzymes is still at the same rate. This affects the synaptic neurotransmission thus causing the death of cells by the production of inflammatory responses [17].

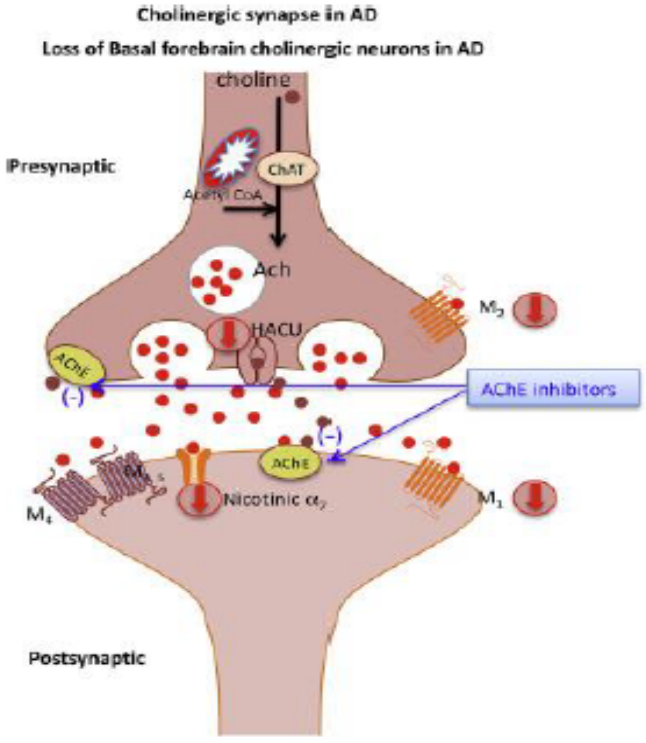


FIGURE 2.3: Cholinergic synapse in Alzheimer disease [17].

2.3.3 Oxidative Stress

Alzheimer disease is also caused by the deposition of metal ions in the brain of the patients. Metal ions such as Cu and Fe stimulate the oxidative stress and are also responsible for accumulation of increase number of plaques in the brain cells [18]. Formation of plaques effect the functioning of microglial cells and astrocytes

in the brain. This can also cause the death of mitochondrial cells resulting in the impairment of mitochondria by the production of reactive oxygen species (ROS) hence causing neurodegeneration.

2.3.4 Genetic Causes

Apolipoprotein E (APOE), a 34 KDa glycoprotein present in long arm of chromosome 19 is present in human beings in three allelic forms (APOE 2, 3, 4). It aids in neuronal repair and growth of dendritic cells. It is also involved in inflammatory processes. However, a rare mutation in APOE-4 can cause an increase in deposition of plaques and tangles also enhancing oxidative stresses and damage to mitochondria [19].

2.4 Types of Alzheimer Disease

The subtypes of Alzheimer's disease are:

2.4.1 Early On-set Alzheimer's

Early on-set Alzheimer is also known as Familial type. It occurs in individuals of age less than 65 years. Patients with early Alzheimer's show a rare mutation in chromosome 14. Early On-set Alzheimer's is related to three main genes Amyloid β precursor protein (APP), Presenilin 1(PSEN-1) and Presenilin 2 (PSEN-2) [20]. Patients are usually diagnosed at the age of 30s to 40s. The symptoms include vision loss, forgetting things and difficulty in completion of daily life tasks.

2.4.2 Late On-set Alzheimer's

Late On-set Alzheimer is the sporadic type. It is a common form of the disease occurring in individuals of age greater than 65 years. These patients show severe

form of symptoms with age also considered as a major risk factor. Tangles and plaques are visible in patients suffering from late Alzheimer's with a mutation in APOE 4 gene. Patients show dysfunction in social, physical and cognitive abilities [21]. The differences in characteristics of Early Onset and Late Onset Alzheimer disease are summarized in **Table 2.1**.

TABLE 2.1: General characteristics of early and late on-set Alzheimer [21].

S.No		**Load**	**Eoad**
1	Age at Onset	65 years & older	Younger then 65 years
2	Form of Onset	Amnestic	Non-amnestic
3	Progression	Slower	Faster
4	Neuro-Physiology	Poorer memory	Poorer executive function & motor skill
5	Pathology Findings	Senile plaques & neurofibrillary tangles	Senile plaques & neurofibrillary tangles with better preservation the hippocampus
6	Biomarkers-in CSF	Lower level of AB42 and increase in tau and P-tau	Lower level of AB42 and increase in tau and P-tau
7	APOE genotype	Favored by 1 or 2 €4 alleles	Favored by absence of €4 alleles

2.5 Prevalence and Incidence

Alzheimer disease has become a global concern. According to the recent data gathered more than 24 million people around the world are suffering from dementia with Alzheimer disease as the leading cause of it. As per the statistics of WHO, US has highest prevalence of Alzheimer disease with an estimate of 9.7%. However 4.4% people in Europe, 4.0% in China, 1.6% in Africa and 1.39% in Pakistan are suffering from Alzheimer disease [22]. In developed countries 1 in every10

people above age 65 is suffering from the disease and more than one third of the old generation show similar signs and symptoms. It is expected that this rate of affected cases will double every 5 years with an approximate increase of 6.7% in each state worldwide between years 2020 to 2025. The number of patients above age 65 is to be double every year reaching to 80 million cases by the year 2040 [23].

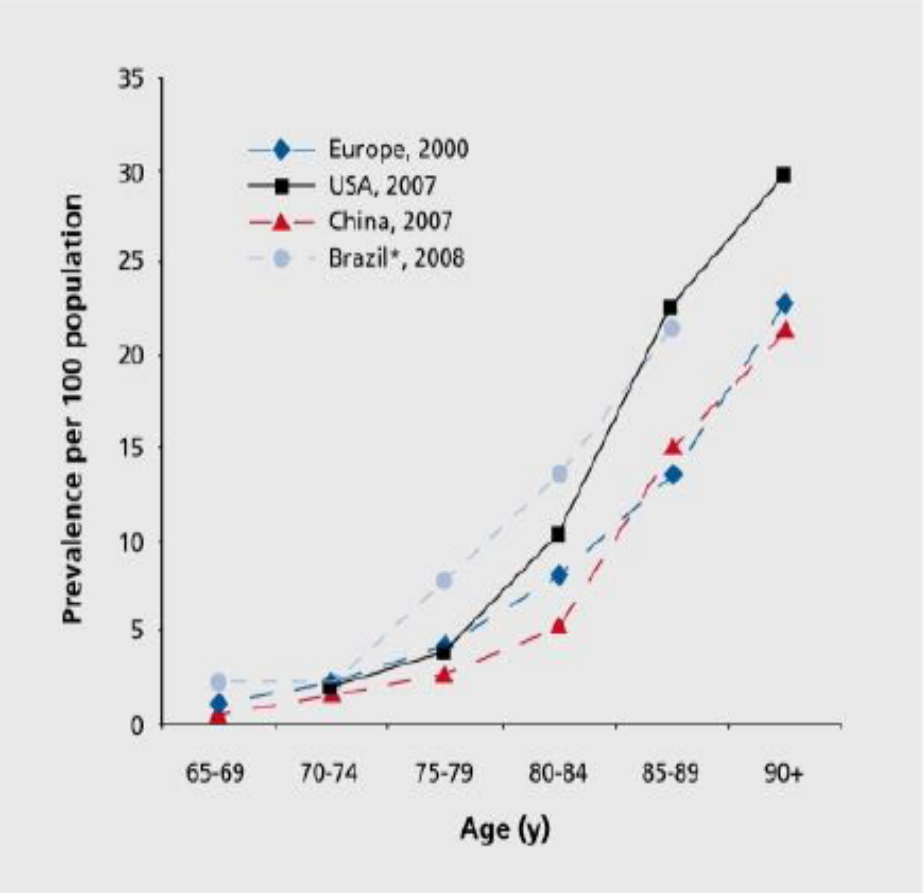


FIGURE 2.4: Prevalence of Alzheimer disease across countries [23].

2.6 Treatment of Alzheimer Disease

Over the past decade, a number of treatment options and techniques are discovered for Alzheimer disease. Both pharmaceutical and therapeutic treatment can be done for Alzheimer's, however the plan of the treatment depends on a number of factors including type of the disease, age of the patient, gender, medications a

patient is already taken along with the individuals overall health and severity of the symptoms. The treatments of Alzheimer disease are described below.

2.6.1 Pharmacological Treatment

There are several drugs that are approved by Food and Drug Administration (FDA) to treat different stages of Alzheimer disease over the years. These drugs work to improve the symptoms and also to target major causes of Alzheimer disease such as inhibiting the cholinergic action and formation of plaques and tangles.

2.6.1.1 Cholinesterase Inhibitors

These are the drugs that improve the cholinergic deficit by preventing the breakdown of Acetylcholine and inhibiting the action of cholinesterase enzymes. Tacrine was the first drug approved in 1993 [24], for the treatment of Alzheimer disease which later paved the way for other drug options.

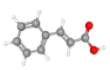
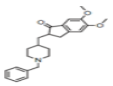
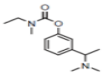
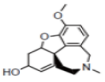
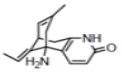
However later on Tacrine showed side effects such as hepatotoxicity. Other drugs approved for the treatment of Alzheimer disease are Donepezil (Aricept), Rivastigmine, (Exelon) Galantamine (Razadyne) and Huperzine A.

Clinical Trials were conducted which indicated that these drugs can reduce the cognitive and behavioral symptoms associated with the disease and improve the functioning ability of patients [25].

Table 2.2 shows the structure of the drug, dosage, target enzymes and side effects. According to a study patient suffering from Mild AD showed preferable reaction to these drugs as compared to the patients suffering from severe form of the disease.

Huperzine A shows stronger inhibition of AChE than other drugs as it can cross Blood Brain Barrier (BBB) providing neuroprotection [26]. Milamine and xanomeline drugs are also used to improve cognitive function by working as agonist for M_1 receptor.

TABLE 2.2: Characteristics of 5 major cholinesterase inhibitors [25].

	Tacrine	**Donepezil**	**Rivasti-Gmine**	**Galant-Amine**	**Huper-Zine/A**
Structure					
Target Enzymes	AChE & BChE	AChE	AChE & BChE	AChE	AChE
Recommended Dosage	160mg/ day	10 mg/ day	9.5 mg/ day	24 mg/ day	0.4 mg/ day
Plasma half life	2-4h	About 70h	About 3h	About 7h	About 60h
Period of disease treatment	——	All stages of Alzheimer disease	Mild to moderate Alzheimer disease	Mild to moderate Alzheimer disease	Mild to moderate Alzheimer disease
AChE IC_{50} (nM)	190	22	48,000	800	47
BChE IC_{50} (nM)	47	4.1	54,000	73,000	30
Adverse Reactions	Hepato-toxicity	Diarrhea, nausea	Diarrhea, nausea	Nausea, weight loss	Nausea

2.6.1.2 Anti Aβ Drugs

Various Calcium antagonists, antioxidants, Nonsteroidal anti-inflammatory drugs (NSAID), Iron chelators and Hypolipidemic drugs such as APoE isomers are being discovered to remove A β plaques that are the major contributor to the progression of Alzheimer disease.

1. Calcium antagonists such as nipodene, flunarizine and verapamil can regulate the blood flow in the brain by inhibiting the overload of calcium in the brain.

2. Seligiline, melatonin and vitamin E are commonly used in clinics to reduce the oxidative action of plaques and prevent cellular death [27].

3. Nonsteroidal anti-inflammatory agents such as aspirin, indomethacin, naproxen and ibuprofen have showed better responses in slowing the progression of Alzheimer disease by helping treat the symptoms.

4. Iron Chelators can be used to prevent neurotoxicity by remove extra amount of iron from the brain. This includes drugs such as desferrioxamine [28].

2.6.1.3 Other Therapies

Various other age related disorders such as diabetes, hypertension, and chronic obesity have also been proved to be related with AD. Intranasal insulin used for the treatment of diabetes 3 has shown to be a possible treatment option for AD as it can cross the blood brain barrier.Anti-depressants such as nortriptyline and desipramine have proved beneficial for treating depression in patients suffering from AD [29].

Statins that help in lowering cholesterol can also prevent dementia. Similarly there is a sudden change in the level of estrogen in women during menopause that can lead to the development of AD. So, by giving the required amount of estrogen and doing estrogen replacement therapy can be effective in delaying the onset and progression of AD.

2.6.2 Non-Pharmacological Treatment

Non-pharmacological treatment is also important for patients suffering from AD to help in cognitive and behavioral disturbances and also be used as prevention from AD. Emotional and physical care is required to help patients cope up with irritability, anxiety and depression from the disease.

Treatment plan of patients also include maintenance of a healthy lifestyle and diet. Aerobic exercises are also an important part as release of neurotropic factors help prevent the cognitive decline.

2.7 Medicinal Plants

Medicinal plants are those plants that have shown remedial properties and have shown beneficial outcomes in both humans and animals. Natural compounds extracted from plants can be used as pharmaceuticals, pesticides, flavor and fragrance ingredients and agrochemicals. Plant components also have the characteristics and ability to prevent the development of certain diseases. From the past several decades these plants are used to cure the ailments of humans. Early humans either applied plants directly on their injuries or complex mixtures of herbs were obtained. Different parts of the plants are used for the development of drugs such as seed, leaf, flower or even the complete plant. As per a recent study, 25% of the total medicines used in this modern era are directly or indirectly prepared by using plants [30]. Plants usually have bioactive compounds such as alkaloids, flavoids, essential oils that can be used for drug development. According to WHO in the modern era, approximately 4 billion people, 80% of the world population uses herbal medicines as primary health care [31]. However the distribution of plants is not fair. Most of the species of plants can only be obtained from wildlife populations.

With the discovery of Alzheimer's in 1906, continuous efforts were started to remedy the disease. Herbal medicines with therapeutic potential were also studied. Even though no exact treatment was inclined but still a number of plant based drugs were produced to relieve the symptoms of the disorder from acute to severe level. In the past years a large variety of plants belonging to different family and their products have been reported to have potential of anti-ChE activity. Different plants such as *Ginkgo biloba*, *Melissa officinalis* and *Salvia officinalis* are used for thousands of years to relieve the symptoms of AD and improve the cognitive performance. Medicinal plants have a promising future for as long as they are existing in nature. Almost half a million plants are present worldwide and most of them are still not studied for their medicinal properties. So the ongoing and future studies on medicinal plants can prove beneficial for the treatment of a wide variety of diseases [32].

2.8 *Ajuga bracteosa*

Labiatae, a plant family plays a crucial role in Medicine. It comprises of 170 genera and 3000 species most of which show medicinal properties. Ajuga is the largest genera of family *labiatae*. The name of the genus *Ajuga* come from a latin word **(Abija)** that means drive away. The name refers to the medicinal properties of the plant. The 301 species of genus *Ajuga* with different variations are spread all over the world and are mostly native to Europe and Asia [33]. Out of these 301 species *Ajuga bracteosa*, commonly known as Bungle in English, kauri booti in Hindi and Jan-i-adam in Kashmiri is a perennial ascending hairy herb as shown in the figure. The plant has sub-spathulate leaves with lobed margins and grows up to 5 to 50 cm in height [34]. The plant has yellowish flowers and is present in the form of axillary spirals. The rootstock is woodier with exerted stamens. *Ajuga bracteosa* is mostly grown in sub-tropical and temperate regions of Kashmir to Bhutan, Afghanistan, India, Pakistan, China, Malaysia and western Himalaya.

Ajuga bracteosa has been used as medicinal plant from late centuries. From the past few decades this plant is used both for traditional purposes and also for Ayurveda preparation. In Ayurvedic system this perennial herb is used for the treatment of gout, rheumatism, palsy and amenorrhea [35]. Different plant parts have shown various uses such as the juice extracted is known for the treatment of dysentery and diarrhea. Plant leaves are used for the treatment of high fevers and in certain parts for India it works as a substitute of quinine for the treatment of malaria, inflammation and diabetes. With this *Ajuga bracteosa* has also shown positive effects as antioxidant, blood purifier, anti-depressant and anti-inflammatory agent.

Ajuga bracteosa is rich in a number of metabolites and its derivatives are also used in medicine. These include terpenoids, flavoids, glycosides, steroids, sitosterols, tannins and essential oils [36]. Due to the presence of these metabolites it is used as an anti-microbial, astringent, anti-asthma and as a cooling agent. The leave extract of the plant can be used to cure headaches, throat and ear infections, coughing and stomach disorders. Plant extracts also showed the ability

to inhibit cholinesterase enzymes such as AChE and BChE and can be used to treat Alzheimer's [37]. However more studies are required to check the toxicity and harmful effects of the plant in use.

FIGURE 2.5: *Ajuga bracteosa* [33].

2.9 Taxonomic Hierarchy

Ajuga bracteosa is the binomial name for plant belonging to family Lamiaceae. It is found all over the world and is native to Europe, Asia, North America and India. It is used to treat a number of disorders.

Ajuga bracteosa is a eukaryotic organism that belongs to kingdom-Plantae, Phylum-Tracheophyta, Class-Magnoliopsida, Order-Lamiales, Family-Lamiaceae, subfamily-Ajugoideae, Tribe-Ajugeae , Genus-Ajuga and specie *A.bracteosa* [38].

2.10 Molecular Docking

Molecular docking is a computational tool used in structural molecular biology. It has been used as an efficient process to predict drug design from the past three decades. The basic goal of this virtual screening process is to predict the bonding of two molecules and their structures. Different biomolecules like protein, enzymes, lipids, and carbohydrates interact with ligands and their structures can be predicted through this process [39]. Docking is a preferable bioinformatics tool for virtual screening of the compounds that are present in libraries and databases for the analysis of their structures, strength of the bond between ligand and targeted proteins, binding affinity and functions.

Molecular docking can be done by different software such as Auto Dock, Auto Dock vina, CB Dock and ICM etc. One or more search algorithms are used for best result prediction of receptor-ligand complex. Molecular docking has gain wide acknowledgement in research areas and have become an important tool for drug designing and other molecular modeling applications. In molecular modeling docking gives scoring function which gives the score for molecule interaction and this accuracy makes this method a success as it help predicts the binding site of the ligands and possible structures. This structural prediction proves beneficial of rational drug design in association of the targeted proteins [40].

2.11 Targetted Proteins

2.11.1 Acetylcholinestrase (AChE)

Acetylcholinestrase also known as AChE, is an important enzyme that belongs to the family of serine hydrolase enzymes. It is the product of gene present on chromosome 7 in human beings. It is found in conducting tissues such as central and peripheral tissues, nerve and motor, sensory cholinergic and noncholinergic fibers. However it also exists in multiple molecular forms in red blood cell membranes.

The major role of AChE is to hydrolyze acetylcholine present in the synaptic cleft into two molecules i.e. Acetate and Choline [40]. The enzymatic reaction is shown in the figure.

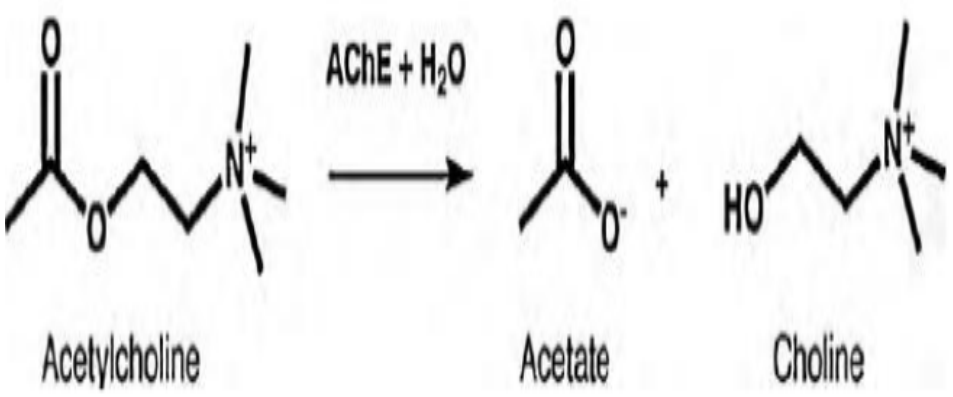


FIGURE 2.6: Enzymatic hydrolysis of Acetylcholine [41].

Acetylcholine has key role in learning and memory processing as it activates the nicotinic and muscarinic receptors of Cental Nervous System (CNS) [42]. The important function of acetylcholinestrase is to inhibit the function of neurotransmitter ACh by the hydrolysis into the constituents at the cholinergic synapses. A single AChE molecule can hydrolyse 2500 molecules of acetycholine.

AChE exists in ellipsoidal form and a single molecule has $\aleph/\beta$ protein. 12 βsheets are surrounded with 14 αhelices. X- crystallography indicates that the enzyme has 20 Å deep gorge catalytic active site (CAS) which has serine, histidine and glutamate residues at the bottom known as Acylation or A site [43]. The active site is divided into two subsites known as anionic or estratic site that work for catalysis and binding pocket for choline respectively. The region near the top rim is Peripheral or P site (PAS) has 10 aromatic residues and plays a role in orientation and binding of substrates including acetylcholine. It has residues such as Tyrosine 70,121 and Tryptophan.

So far three crystal structures of AChE have been reported. These include human AChE (hAChE), mouse AChE (mAChE) and Torpedo California AChE (TcAChE). The dimers in these species overlap and are similar with two opposite positioned subunits that form a four helix bundle [44].

2.11.2 Butyrylcholinestrase (BChE)

Butyrylcholinestrase also known as pseudo-cholinestrase is also an important serine hydrolase enzyme that hydrolyses many choline based esters. It is the product of gene present in chromosome 3 in human beings. It is present in blood plasma of the humans and is produced in the liver. BCHE gene encodes the enzyme. The overall function of BChE is similar to AChE and shares 65% structural homology [45].

BChE binds with ligands with different specificity due to difference in number of aromatic residues in the active site. It contains Tyr332 in P-site and Trp82, Phe329 and Trp 231 in A- site.

2.12 Natural compounds as inhibitors of AChE and BChE

Cholinestrase enzymes (AChE and BChE) hydrolyze the acetylcholine in the brain causing the cognitive defects leading to Alzheimer. Inhibition of these enzymes can alleviate the symptoms. The 3D structure of the cholinesterase enzymes were screened and a number of plant based compounds were identified that can bind to the enzyme active site and have potential to show inhibitory effects to the enzyme. Three natural AChEi alkaloids Rivastigmine, Galanthamine and Huperzine A were approved by FDA for treatment of cognitive loss in Alzheimer patient. Several other phytochemicals including 3-hydroxy-2,2-6-trimethyl-3,4,5,6-tetrahydro-2H-pyrano [3,2/c] quinoline-5-one, ribalinine and methyl isoplatydesmine isolated from the plant *Skimmia laureola* with a Ki of 110, 30 and 30 μM , N-methylasimilobine from the plant *Nelumbo nucifera*, isoquinoline alkaloid stylopine, epiberbine from tuber of the plant *C.turtschaninovii*, groenlandicine from rhizomes of the plant *Coptis chinensis*, skimmianine from the plant *Zanthoxylum nitidum*, coronaridine from the plant *Ervatamia hainanensis* showed inhibition of cholinesterase enzymes [46]. Six labdane-type diterpeniods were extracted from the plant Leonurus het-

erophyllus by bioassay fractionation. Leoheteronin A and leopersin G have 15, 16 epoxy group and were identified to be strong inhibitors of cholinesterase enzymes with IC50 values of 11.6 and 12.9 μM respectively [47].

In recent studies several other phytochemicals such as steroids, alkaloids, anthocyanin, flavonol glycosides and triterpenoids which were derived from plant Convolvulus pluricaulis were highlighted to have anti-tumor and anti-inflammatory activities which can work to enhance memory and reduce the symptoms of AD [48]. Many other natural compounds from plants like Valeriana officinalis, Punica granatum L, Salvia officinalis, Bacopa floribunda, Jatropha carcus, Mirabilis jalapa, Canna indica and many more plant derivatives have shown effects to improve memory and work as inhibitor for cholinesterase enzymes [49].

2.13 Inhibitors Against AChE and BChE in *Ajuga bracteosa*

A variety of naturally occurring compounds have shown potential to serve as anti-tumor to inhibit the activity of enzymes in Alzheimer. These compounds are beneficial for use as they have minimum side effects and are easily available to a large population. Traditionally, *Ajuga bracteosa* was used either in the form of juice or tea and the herb is known to cure malaria, gout, dyspepsia, and other fevers. Besides these the compounds from *Ajuga bracteosa* are well known for their anti-inflammatory, anti-microbial and anti plasmodial activity [50].

Different metabolic compounds including sterols, diterpenoids, glucosides, ethanol, n- hexane, withanolides, triglycerides, and phytoecdysteroids are obtained from roots, oil and leaves of the plant. These compounds have shown positive effects for inhibition of AChE and BChE, but still the exact role is to be studied. And also the side effects and toxicity effects against the use of the plant are to be tested.

Chapter 3

Materials and Methods

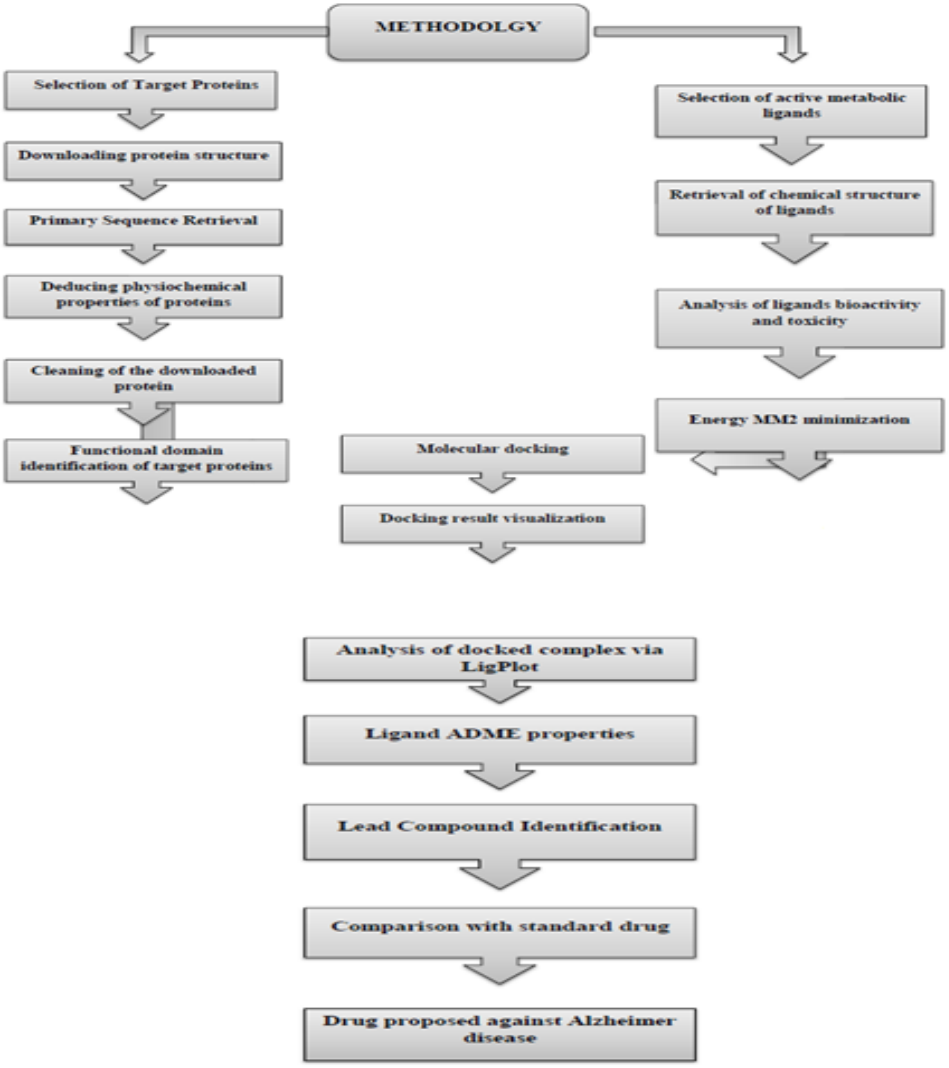


FIGURE 3.1: Overview of Methodology.

3.1 Disease Selection

Alzheimer disease is a neurological condition and a common form of dementia that begins with a mild memory loss and slowly leads to the inability of a person to do simples tasks of daily life. It has a global prevalence of as high as 24 million with an estimate of this ratio to triple in next ten to twenty years.

For this extreme panic research for targeting the major cause are crucial. To control the progression of the disease the availability of the drugs is to be ensured. Acetylcholine deficit was identified to play a major role in the development of the disease. Acetylcholinestrease and other novel enzymes are involved in degeneration of acetylcholine [51]. For this purpose it provides a potential site for drug targeting. Despite the continuous efforts gaps are still present which need to be filled.

3.2 Selection of Target Proteins

The receptive proteins were selected as they played a vital role in pathogenesis of Alzheimer disease. Acetylcholinestrase (AChE) and Butyrylcholinestrase (BChE) are the two main neurotransmitters that break down acetylcholine in synaptic cleft into acetate and choline compounds. The 3D crystalline structure of AChE and BChE were downloaded from the available resource Protein Data Bank (PDB). The Protein Data Bank is a data base for the three dimensional structural data of large biological molecules such as proteins and nucleic acids. These human specific proteins have codes 4M0E with DOI 10.2210/pdb4M0E/pdb and 5NN0 with DOI 10.2210/pdb5NN0/pdb respectively in PDB.

3.3 Primary Sequence Retrieval

The primary sequence of target proteins (4M0E and 5NN0) were downloaded in FASTA format from a computational tool UniProt under accession number P22303 and P06276 respectively.

3.4 Deducing of Physiochemical Properties of proteins

Physiochemical properties are important for determining the function of a protein. For this purpose ProtPram, a computational tool of ExPAsy was used. Physiochemical properties liker molecular weight, isoelectric point, number of amino acids present;

Ext. coefficient (Cys included), Ext. coefficient (Cys not included) , grand average of hydropathicity (GRAVY), aliphatic index, instability index, number of positively charged residues (Arg+ Lys) and negatively charged resideues (Asp+ Glu) were computed.

3.5 Cleaning of the Downloaded Protein

After downloading the structure, the extra constituents attached to the protein were removed by using an open source system PyMOL. AChE consisted of 1-542 amino acids and was referred as A and B chain.

BChE contains 1-529 amino acids and has a single Linear A chain. All remaining constituents attached to the protein were removed for an efficient processing.

3.6 Functional Domains Identification of the Target Proteins

Interpro, an online database was used to analyze the functional domain and sites of AChE and BChE. By inserting the FASTA sequence of the proteins in the data base polypeptide binding sites and homodimer interfaces were studied.

3.7 Selection of Active Metabolic Ligand

Active metabolic ligands from the medicinal plant *Ajuga bracteosa* were selected. These ligands have previously shown antitumor and anti-microbial properties. These included terpenoids, flavoids, glycosides, steroids, esters, phenols, sitosterols, tannins and essential oils [35].

3.8 Retrieval of Chemical Structure of Ligands

Ligands of the selected plant were searched from PubChem, which is the World's largest repository of chemical information database [52]. PubChem is maintained by the National Center for Biotechnology Information (NCBI).

It contains information of biological molecules i.e carbohydrates, proteins, lipids, modified macromolecules stored in the form of chemical names, molecular formulas, 2 and 3 dimensional structures, their isomers and canonical similes [53].

The structures of ligands obtained from PubChem were downloaded and MM2 energy was optimized. The structures of all ligands were stored in SDF format.

3.9 Analysis of Ligands Bioactivity and Toxicity

Chemical compounds used as ligands were virtually analysed on the bases of Lipinski rule of five as it sets the criteria for any compound to be used as an active drug in humans [54].

The effectiveness of a compound is measured by its drug like ADMET properties. pkCSM is an online tool used to find the ADMET properties of compounds. The rules are as follows:

- The log P value of a drug-like compound must be limited to 5.
- Molecular weight should not exceed 500.
- Maximum number of H-bond donor should be 5.
- Maximum number of H-bond acceptor should be 10.

3.10 Molecular Docking

Molecular docking is a method used to predict the most favorable conformational interaction between selected ligands and target proteins. For performing the molecular docking CB-dock (Cavity detection guided protein-ligand blind docking) was used. CB-dock automatically predicts the binding regions of a protein and by using a curvature based cavity detection method calculates the size and the center [55].

After adjusting the box size according to the ligand, docking was performed using AutoDock Vina. The 3d structure of protein in pdb format and 3d structure of ligand in sdf format was uploaded and docking was performed.

The result provided by CB- dock was in 5 different poses of interaction, among which the best one was selected on the basis of size of cavity and minimum vina score in KJ/m-1 [56].

3.11 Docking Result Visualization

The image of the output from docking was generated using PyMOL. PyMOL is a molecular graphic tool used to visualize the three dimensional structure of proteins, nucleic acids, electron densities, surfaces and trajectories.

Pymol provides a plugin that gives a clear picture of the docking result and make its visualization easier [57]. The docking result was saved in pdb format and then visualized via PyMOL and then saved in pdb format.

3.12 Analysis of Docked Complex via Ligplot

Docked complex obtained in pdb format with the lowest vina score was analyzed using the software LigPlot. The schematic diagrams of protein and ligand interactions in the given pdb format were generated automatically. The interactions were modified by hydrogen bond and through hydrophobic contacts. The diagrams generated through LigPlot illustrated hydrogen bond and hydrophobic interactions between ligands and main or side chains of the proteins were indicated as dashed lines and arcs between atoms [58].

3.13 Ligand ADME Properties

After the analysis the compounds were further screened for ADMET properties. pkCSM was used to optimize ADMET properties which are adsorption, distribution, metabolism, excretion and toxicity related to the human body.

3.14 Lead Compound Identification

After the analysis of protein-ligand interactions, docking scores and toxicity studies, the most active inhibitor that fulfills Lipinski's rule of five was selected. This selected compound was our lead compound.

3.15 Comparison with Standard Drug

Donepezil (sold under the name Aricept) which can be taken in all stages of Alzheimer disease was selected as a standard drug for comparison against the lead compound. Though it is known to improve cognitive performance and functional ability in patients suffering from Alzheimer's but the frequent use of it can lead to a number of side effects such as anorexia, dizziness, nausea [59].

Chapter 4

Results and Discussions

4.1 Structure Modeling

Target proteins AChE and BChE were selected as drug candidates against bioactive constituents present in *Ajuga bracteosa*. These components include phenols (hydroquinone, resorcinol and pyrocatechol), diterpenoids (β sitosterols), glycosides (ceryl alcohol, cerotic acid and palmitic acid), tannins (ellagic acid and ferulic acid).

Ecdysteroids (pthalic acid, ajugalactone and 20-hydroxyecdysone), flavanols (quercetin), essential oil products (camphene, elemol, α humulene, β mycrene) and cinnamic acid derivatives (caffeic acid, chlorogenic acid, pcoumaric acid and transcinnamic acid).

4.1.1 Sequence Retrieval of Protein

The Primary sequence of target proteins (AChE and BChE) was downloaded in fasta format from Uniprot using the accession number P22303 and P06276 respectively.

>sp—"P22303—ACES-HUMAN OS=Homo sapiens OX=9606 GN=ACHE PE=1 SV=1

MRPPQCLLHTPSLASPLLLLLLWLLGGGVGAEGREDAELLVTVRGGRLR GIRI LKTPGGPVSAFLGIPFAEPPMGPRRFLPPEPKQPWSGVVDATTFQS VCYQYVI DTLYPGFEGTEMWNPNRELSEDCLYLLHYTDWLHPEDPARLRE ALSDVVGDH NVVCPVAQLAGRLAAQGARVYAYVFEHRASTLSWPLWMGV PHGYEIEFIFGIP LDPSRNYTAEEKIFAQRLMRYWANFARTGDPNEPRD PKAPQWPPYTAGAQQY VSLDLRPLEVRRGLRAQACAFWNRFLPKLLSAT DTLDEAERQWKAEFHRWSSI YMVHWKNQFDHYSKQDRCSDL"

>sp—"P06276—CHLE-HUMAN OS=Homo sapiens OX=9606 GN=BCHE PE=1 SV=1

DIIIATKNGKVRGMNLTVFGGTVTAFLGIPYAQPPLGRLRFKKPQSLTKWSDIWN ATKYANSCCQNIDQSFPGFHGSEMWNPNTDLSEDCLYLNVWIPAPKPKNATVLIW IYGGGFQTGTSSLHVYDGKFLARVERVIVVSMNYRVGALGFLALPGNPEAPGNMG LFDQQLALQWVQKNIAAFGGNPKSVTLFGESAGAASVSLHLLSPGSHSLFTRAILQ SGSFNAPWAVTSLYEARNRTLNLAKLTGCSRENETEIIKCLRNKDPQEILLNEAFVV YGTPLSVNFGPTVDGDFLTDMPDILLELGQFKKTQILVGVNTQNNSTSWPVFKIMI KLRAQQKTMMREMTGNIDEAEWEWWNNYMMDWKNQFNDYTSKKESCVGL"

4.1.2 3D Structure of Protein

The target proteins chosen are acetylcholinestrase (AChE) and butyrylcholinestrase (BChE). The 3D structures of proteins AChE and BChE were obtained from protein data bank (PDB). An online database for structural data of large molecules named as 4M0E with the DOI 10.2210/pdb4M0E/pdb and 5NN0 with DOI 10.2210/pdb5NN0/pdb respectively. AChE obtained was in complex with dihydrotanshinone as shown in **Figure 4.1** and BChE was attached to 2 napthamide as shown in **Figure 4.2**. These groups need to be removed for further processing.

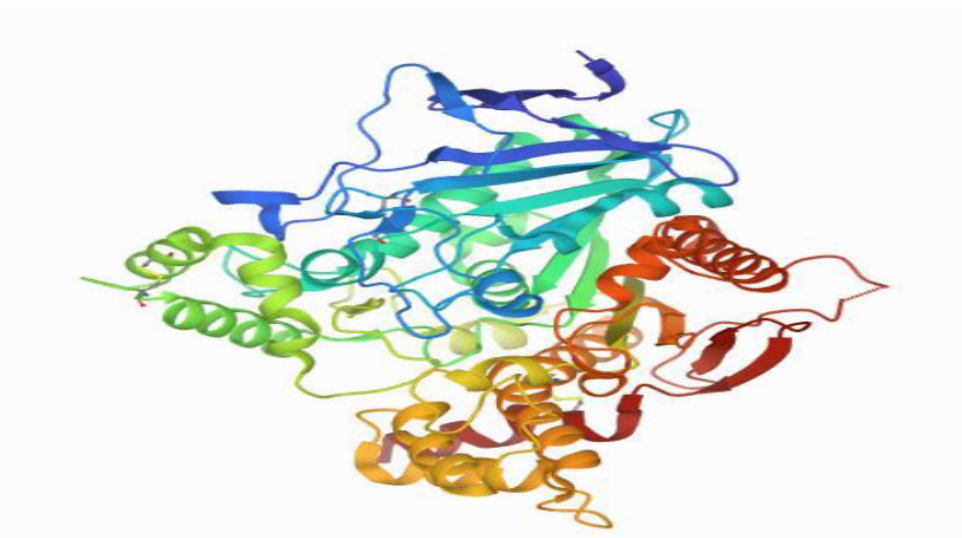


FIGURE 4.1: 4M0E complexed with dihydrotanshinone.

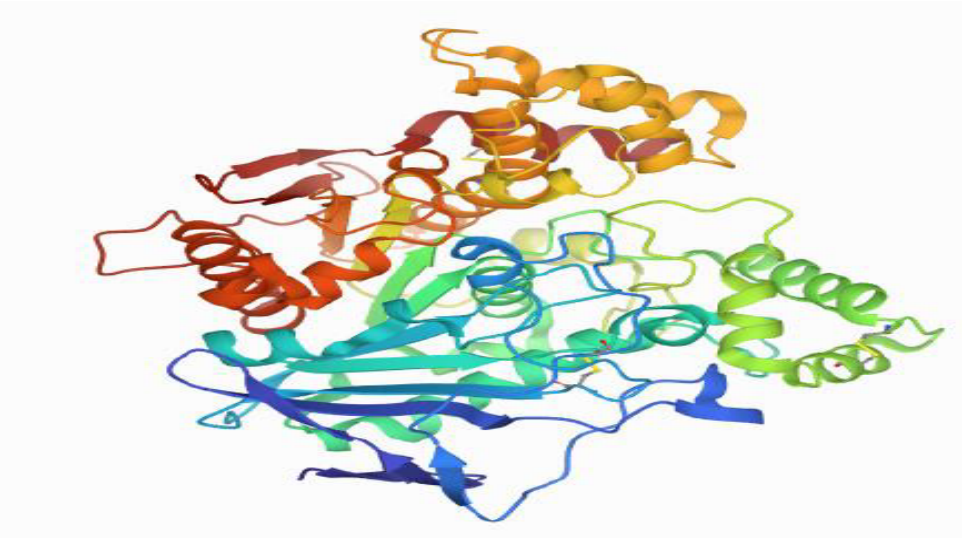


FIGURE 4.2: 5NN0 complexed with 2 napthamide.

4.1.3 Physiochemical Characterization of AChE and BChE

A tool of ExPASY named as ProtParam was used to study the properties of proteins AChE and BChE. It is an online program used to compute different physical and chemical properties of proteins stored in Swiss-prot or TrEMBL or for the sequence of proteins that are entered by users. The parameters computed include molecular weight, atomic composition, proteins amino acid composition,

estimated half-life, extinction co efficient, instability index, theoretical pI, aliphatic index and lastly grand average of hydropathicity (GRAVY). The pI of the protein represents acidity and basicity values. pI greater means protein is basic in nature and less than 7 shows the acidic nature. Extinction coefficient shows absorption of light whereas instability index represents stability level of protein if it is lesser than 40 means protein is stable and values greater than 40 shows instability of protein [60].

The aliphatic index shows the aliphatic content of protein. The high level indicates the thermo stability of a protein. The molecular weight (MW) represents the values of both positive and negatively charged amino acid residues. PR shows positively charged residues (Arg+Lys) and NR indicates negatively charged residues (Asp+Glu). Low GRAVY shows better interaction of water molecules. All the above parameters were taken into consideration while performing research work. The physiochemical properties of the selected protein AChE and BChE are shown in Table 4.1 and 4.2 respectively.

Results from Table 4.1 indicate that Acetylcholinestrase has a combined molecular weight of both positive and negative amino acids as 59390.46. The pI value indicates that the target protein is slightly acidic. Protein is thermostable as per the aliphatic index. The low value of GRAVY shows that protein has better interaction with water molecules.

TABLE 4.1: Physical properties of AChE.

S No	**Parameters**	**AChE**
1	M.W	59390.46
2	pI	5.83
3	NR	54
4	PR	46
5	Ext.Co 1	100185
6	Ext.Co 2	99810
7	Instability Index	40.07
8	Aliphatic index	84.00

9	GRAVY	-0.110

Results from **Table 4.2** indicate that Butyrylcholinestrase has a molecular weight of 59639.84 which is combined molecular weight of both positive and negative amino acids. The pI value indicates that the target protein is basic. The aliphatic Index showed that the protein is thermostable. The low value of GRAVY shows that protein has better interaction with water molecules.

Table 4.2: Physical properties of BChE.

S No	**Parameters**	**BChE**
1	M.W	59639.84
2	pI	7.22
3	NR	53
4	PR	53
5	Ext.Co 1	104195
6	Ext.Co 2	103820
7	Instability Index	37.63
8	Aliphatic index	77.60
9	GRAVY	-0.275

4.1.4 Identification of Functional Domains of the Proteins

Functional domain of proteins can be identified using InterPro. Proteins can have more than one domain performing different functions. Functional domains are major part of a protein and are sites utilized by proteins to interact with other protein or other substances. InterPro is an online database of protein families that helps in functional analysis of proteins and classifies them into families by identifying domains and other important sites.

The job ID for finding functional domain of 4M0E is https://www.ebi.ac.uk- /interpro-/result/InterProScan/iprscan5-R20220505-164308-0580-86253863-p1m/.

And for 5NN0 is https://www.ebi.ac.uk/interpro/result/InterProScan/iprscan5-R20220-505-163627-0701-40909301-p2m/.

FIGURE 4.3: Functional domain of targeted protein AChE.

FIGURE 4.4: Functional domain of targeted protein BChE.

4.1.5 Protein Structure Refined for Docking

The protein structure was refined by using PyMol. Dihydrotanshinone molecule in 4M0E and 2-napthamide inhibitor in 5NN0 were removed as shown in **Figure 4.5 and 4.6** respectively, now the protein is ready for docking.

FIGURE 4.5: 4M0E cleaned protein.

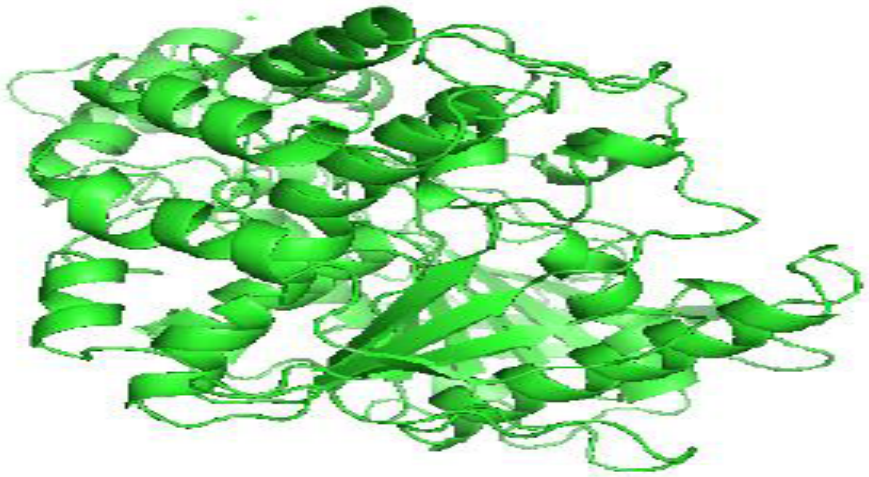


FIGURE 4.6: 5NN0 cleaned protein.

4.2 Ligand Selection

The PDB (Protein data Bank) contains abundant data related to protein ligand complexes. For this reason, the selection of ligand was based on its resolution

structure with chemical class of the protein and their best binding affinities. This selection process required selective binding of ligand with the conformer strengthening it and increasing its population with respect to the population of the protein.

AChE and BChE are main neurotransmitters found in synaptic cleft that control the hydrolysis of Acetylcholine. Because of this major role they have been proven to be excellent targets for drug discovery against Alzheimer's [61]. Several bioactive compounds obtained from *Ajuga bracteosa* show potential targets against receptor proteins AChE and BChE. These inhibitory compounds were searched from world's largest chemical databank-PubChem (https://pubchem.ncbi.nlm.nih.gov). The 3D structures of these ligands were downloaded in sdf format.

After downloading the structures of selected ligands the energy of ligands was minimized in the next step. This is important step as simple downloaded structures of the ligands cant be used because the instability of ligands can affect the vina scores while docking.

TABLE 4.3: Selected ligands with structural information

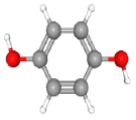
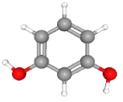
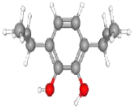
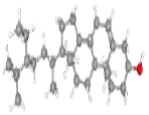
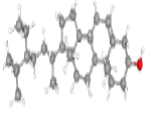
S.No	**Compounds**	**Molecular Formula**	**Molecular Weight**	**Structure**
1	Hydroquinone	$C_6H_4OH_2$	110.11 g/mol	
2	Resorcinol	$C_6H_6O_2$	110.11 g/mol	
3	Pyrocatechol	$C_{12}H_{18}O_2$	194.27 g/mol	
4	β-sitosterols	$C_{29}H_{50}O$	414.7 g/mol	
5	Ceryl alcohol	$C_{26}H_{54}O$	382.7 g/mol	

TABLE 4.4: Selected ligands with structural information

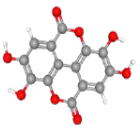
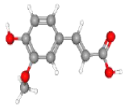
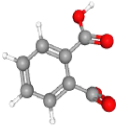
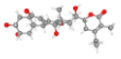
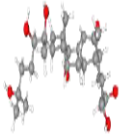
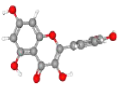
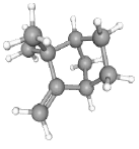
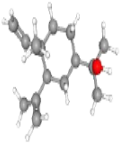
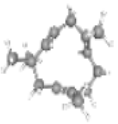
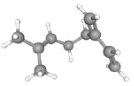
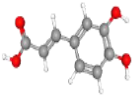
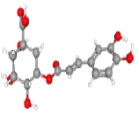
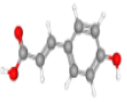
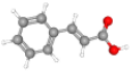
S.No	Compounds	Molecular Formula	Molecular Weight	Structure
6	Cerotic acid	$C_{26}H_{52}O_2$	396.7 g/mol	
7	Palmitic acid	$C_{16}H_{32}O_2$	256.42 g/mol	
8	Ellagic acid	$C_{14}H_6O_8$	302.19 g/mol	
9	Ferulic acid	$C_{10}H_{10}O_4$	194.18 g/mol	
10	Pthalic acid	$C_8H_6O_4$	166.13 g/mol	
11	Ajugalactone	$C_{29}H_{40}O_8$	516.6 g/mol	
12	20-hydroxyecdysone	$C_{27}H_{44}O_7$	480.6 g/mol	
13	Quercetin	$C_{15}H_{10}O_7$	302.23 g/mol	
14	Camphene	$C_{10}H_{16}$	136.23 g/mol	
15	Elemol	$C_{15}H_{26}O$	222.37 g/mol	

TABLE 4.5: Selected ligands with structural information

S.No	Compounds	Molecular Formula	Molecular Weight	Structure
16	α-humulene	$C_{15}H_{24}$	396.7 g/mol	
17	β-mycrene	$C_{10}H_{16}$	136.23 g/mol	
18	Caffeic acid	$C_9H_8O_4$	180.16 g/mol	
19	Chlorogenic acid	$C_{16}H_{18}O_9$	354.31 g/mol	
20	Pcoumaric acid	$C_9H_8O_3$	164.16 g/mol	
21	Transcinnamic acid	$C_9H_8O_2$	148.16 g/mol	

4.3 Virtual Screening and Toxicity Prediction through Lipinski Rule of Five

Bioactive compounds were used as drugs for the disease. For a compound to be considered as drug like or non-drug like, Lipinski rule of five and ADME properties are followed [62]. The Lipinski rule of five is used to determine if a certain chemical compound shows pharmacological characteristics. It deals with certain parameters

like Molecular weight should be 500, log P value 5, H-bond donors 5, H-bond acceptors 10. These rules are to be followed for orally active compounds. The drug like compound is dependent on the method of administration [63].

As per this rule a compound is considered to be drug like if it follows 3 or more rules but violation of 2 or more rules indicates poor absorption and permeation [64].

Table 4.6 shows the applicability of Lipinski rule, Log P value, Molecular weight, H-bond donor and H-bond acceptor values for selected ligands.

TABLE 4.6: Applicability of Lipinski Rule on Selected Ligands

S.No	**Ligands**	**logP Value**	**Molecular Weight**	**H-Bond Acceptor**	**H-bond Donor**
1	Hydroquinone	1.097	110.11 g/mol	2	2
2	Resorcinol	1.097	110.11 g/mol	2	2
3	Pyrocatechol	3.344	194.27 g/mol	2	2
4	β sitosterols	8.024	414.7 g/mol	1	1
5	Ceryl alcohol	9.361	382.7 g/mol	1	1
6	Cerotic acid	9.453	396.7 g/mol	1	1
7	Palmitic acid	5.552	256.42 g/mol	1	1
8	Ellagic acid	1.31	302.19 g/mol	4	8
9	Ferulic acid	1.498	194.18 g/mol	2	3
10	Pthalic acid	1.083	166.13 g/mol	2	2

TABLE 4.7: Applicability of Lipinski Rule on Selected Ligands

S.No	**Ligands**	**logP Value**	**Molecular Weight**	**H-Bond Acceptor**	**H-bond Donor**
11	Ajugalactone	2.163	516.6 g/mol	4	8
12	20 hydroxy-cdyone	1.854	480.6 g/mol	6	7
13	Quercetin	1.988	302.23 g/mol	5	7
14	Camphene	2.998	136.23 g/mol	0	0
15	Elemol	3.942	222.37 g/mol	1	1
16	α humulene	5.035	204.35 g/mol	0	0
17	β mycrene	3.475	136.23 g/mol	0	0
18	Caffeic acid	1.195	180.16 g/mol	3	3
19	Chlorogenic acid	-0.645	354.31 g/mol	6	8
20	Pcoumaric acid	1.49	164.16 g/mol	2	2
21	Transcinnamic acid	1.784	148.16 g/mol	1	1

4.3.1 Toxicity Prediction

PkCSM is an online tool used to calculate the ADMET (Adsorption, Distribution, Metabolism, Excretion and Toxicity) values of the bioactive constituents and drugs. Toxicity of different selected compounds can be calculated by using these tools, for this different methods are used to test if a ligand is toxic or not. AMES

Toxicity (Salmonella typhimurium reverse mutation assay) uses bacteria to find the mutagenic potential of the compound. Positive response indicates that ligand is mutagenic in the DNA of test organism and can also act as a carcinogen. T. Pyriformis toxicity method uses T. Pyriformis (protozoa bacteria) toxicity as a toxic endpoint. Any value > -0.5 log ug/L indicates toxicity. Minnow toxicity test is considered important for risk assessment and hazard calculation of aquatic environment. The values predicted in this test indicate the concentration at which the compound can cause death of 50% Minnows (small bait fishes). Values below 0.5 mM represent acute toxicity. MRTD (maximum recommended tolerated dose) values estimate the toxic threshold of a chemical in a human. It indicates the starting dose of a certain chemical at clinical phase I. Values 0.477 log mg/kg/day is low and value above is considered as high. Hepatotoxicity indicates the liver injury caused by a drug and is an important consideration during drug development. Skin test predicts whether a drug can cause adverse reactions to the skin or not. Oral rat chronic test of toxicity predicts the log value of lowest observed hazardous effects in log mg/kg-bw/day which indicates the concentration of the compound given with that requires the treatment time. The hERG I and II inhibitor test indicates the ability of any compound to inhibit the potential channels that are associated with hERG. A compound inhibiting these channels can potentially cause QT syndrome and person can develop ventricular arrhythmia [65].

The toxicity predicted values of selected ligands is shown in **Table 4.8**. The toxicity values of hydroquinone, resorcinol, pyrocatechol, β sitosterol and Quercetin are given in the table below. The Table shows that all five compounds are non-carcinogenic. β sitosterol, among these five compounds show lowest maximum tolerated dose in humans. All three compounds, except β sitosterol and Quercetin shown in the table below are sensitive to skin and hydroquinone, Quercetin and pyrocatechol also have high MRTD values. All these compounds are not hepatotoxic and have no harmful effects on liver. The ligands are considered toxic against T. pyriformis but show non toxicity for minnows. The Table below shows that β sitosterol can inhibit hERG II. The toxicity values of Ceryl alcohol, Cerotic acid, Palmitic acid, Ellagic Acid and Ferulic Acid are given below. The Table

shows that all three compounds, except ellagic and ferulic acid are sensitive to skin, ceryl alcohol is also hERG II inhibitor whereas cerotic acid is also hepatotoxic. All these ligands show T. pyriformis toxicity but are considered nontoxic for minnows. Ferulic acid has high MRTD value.

The toxicity values of Pthalic Acid, Ajugalactone and 20-hydroxyecdysone are given below. All three compounds are non-carcinogenic and are not sensitive to skin. The ligands shown are not hepatotoxic and have no harmful effects on liver. The table shows that ajugalactone and 20-hydroxyecdysone have low Max.tolerated dose whereas Pthalic acid has high MRTD value. All three compounds give T. pyriformis toxicity but show no hazardous effects against minnows. As indicated in the table, these ligands are not inhibitors of hERG I and II. Toxicity values of camphene, α humulene, β Mycrene and elemol are shown in Table below.

The values indicate that all four compounds are non-carcinogenic and are not hepatotoxic. All ligands, camphene, elemol, β Mycrene and α humulene are toxic against T. pyriformis but are non-toxic against humans, however, elemol and α humulene have skin sensitization. Both, β Mycrene and α humulene compounds have high Max.tolerated dose and are considered toxic for minnows. These compounds are non-carcinogenic and non-hepatotoxic.

The values given in Table below indicate that caffeic acid, pcoumaric acid and transcinnamic acid have high MRTD values whereas chlorogenic acid has low value and is a supporter of potassium channels. All of these compounds are insensitive to skin and are not hepatotoxic hence cause no liver damage. The ligands show T. pyriformis toxicity but cause no toxicity to minnows. All these compounds as mentioned in the table are not inhibitor of hERG I and II.

The toxicity values mentioned in the table shows that on the basis of toxicity tests like skin sensitization, hERG II inhibitor, Minnow toxicity we can screen out hydroquinone, resorcinol, pyrocatechol, β sitosterol , ceryl alcohol, cerotic acid, palmitic acid, elemol and α humulene. All other ligands pass the toxicity test, but final screening would be based on overall ADME properties.

Table 4.8: Toxicity properties of selected ligands.

Ligands	AMES toxicity	Max. tolerated dose (human)	hERG I Inhibitor	hERG II Inhibitor	Oral rat acute toxicity	Oral rat chronic toxicity	Hepato-tocicity	Skin sensi-tization	T. pyri-formis-toxicity	Min-now toxicity
Hydro-quinone	No	0.707	No	No	2.008	2.332	No	Yes	0.105	2.194
Resor-cinol	No	-0.017	No	No	2.14	2.313	No	Yes	0.105l	2.194
Pyrocat-echol	No	1.006	No	No	2.282	2.049	No	Yes	1.451	0.914
β sito-sterol	No	-0.621	No	Yes	2.552	0.855	No	No	0.43	-1.802
Quercetin	No	0.499	No	No	2.471	2.612	No	No	0.288	3.721
Ceryl-alcohol	No	-0.396	No	Yes	1.826	0.829	No	Yes	0.344	-3.337
Cerotic-acid	No	-0.651	No	No	1.405	3.953	Yes	Yes	0.299	-3.492
Palmitic-acid	No	-0.708	No	No	1.44	3.181	No	Yes	0.84	-1.083
Ellagic-acid	No	0.476	No	No	2.399	2.698	No	No	0.295	2.11
Ferulic acid	No	1.082	No	No	2.282	2.065	No	No	0.271	1.825

Continue Table 4.8: Toxicity properties of selected ligands.

Ligands	AMES toxicity	Max. tolerated dose (human)	hERG I Inhibitor	hERG II Inhibitor	Oral rat acute toxicity	Oral rat chronic toxicity	Hepato-tocicity	Skin sensi-tization	T. pyri-formis-toxicity	Min-now toxicity
Pthalic-acid	No	0.582	No	No	1.449	2.165	No	No	0.281	2.378
Ajugala-ctone	No	-0.967	No	No	2.582	1.99	No	No	0.285	2.635
20hydroxy ecdysone	No	-0.214	No	No	2.671	2.174	No	No	0.285	3.492
Camp-hene	No	0.305	No	Yes	1.554	2.247	No	No	0.533	1.19
Elemol	No	0.283	No	No	1.686	1.229	No	Yes	1.921	0.543
α hum-ulene	No	0.551	No	No	1.766	1.336	No	Yes	1.451	0.716
β Myc-rene	No	0.617	No	No	1.643	2.406	No	No	0.894	0.736
Caffeic-acid	No	1.145	No	No	2.383	2.092	No	No	0.293	2.246
Chloro-genic acid	No	-0.134	No	No	1.973	2.698	No	No	0.285	5.741
Pcoum-aric acid	No	1.111	No	No	2.155	2.534	No	No	0.319	1.607
Transcin-amic acid	No	1.11	No	No	2.094	2.651	No	No	0.247	1.825

4.4 Molecular Docking

Molecular docking is a bioinformatics tool used to determine bond strength between ligand and protein at atomic level. It uses vina scoring function to estimate the structure of the ligand that attaches with the binding site of the receptor. The 3D structure of ligand and protein are taken as input for docking. CB Dock, an online blind docking tool is used for this purpose. CB Dock predicts the binding sites of proteins and calculates the cavity size by using a program called Auto Dock Vina. The result and time of docking is based on structure of ligand, receptors and refinement. After docking CB Dock provides the output as five best poses and models for receptors. The best pose among these is selected on the basis of vina score and cavity size. Molecular docking was performed by using AChE and BChE as receptor proteins whose structures were inserted in PDB format. And 21 compounds mentioned above from plant Ajuga bracteosa were selected as ligands. There structures were inserted in sdf format. CB Dock checks these files and uses OpenBabel and MGL tools to convert them into pdbqt format files. After that the program estimated the cavities of receptor and calculated center sizes of top five cavities. The best conformation among these five was selected on the basis of higher affinity scores of receptor- ligand interaction. Table 4.9 and 4.10 shows the interaction of ligands with AChE receptor. Among the selected ligands, quercetin show the highest binding score (-10 kcal/mol) followed by chlorogenic acid (-9.7 kcal/mol). Hydroquinone has the lowest binding score of -5.5 kcal/mol.

TABLE 4.9: Docking results of hydroquinone, resorcinol, pyrocatechol, β sitosterols, ceryl alcohol, cerotic acid and palmitic acid with AChE receptor.

S.No	**Ligands**	**Binding Score (kJ/m-1)**	**Cavity size**	**Grid Map**	**HBA**
1	hydroquinone	-5.5	900	-42	2
2	resorcinol	-5.7	1506	-54	2
3	pyrocatechol	-7.5	1506	-54	2
4	β sitosterols	-8.2	1208	-50	1
5	ceryl alcohol	-6.7	1506	-54	1
6	cerotic acid	-6.7	1506	-54	1
7	palmitic acid	-6.9	1506	-54	1

Continue TABLE 4.9: Docking results of hydroquinone, resorcinol, pyrocatechol, β sitosterols, ceryl alcohol, cerotic acid and palmitic acid with AChE receptor.

S.No	**Ligands**	**HBD**	**LogP**	**M.W (g/mol)**	**Rotatable Bond**
1	hydroquinone	2	1.097	110.11	0
2	resorcinol	2	1.097	110.11	0
3	pyrocatechol	2	3.344	194.27	2
4	β sitosterols	1	8.024	414.7	6
5	ceryl alcohol	1	9.361	382.7	24
6	cerotic acid	1	9.453	396.7	24
7	palmitic acid	1	5.552	256.42	14

TABLE 4.10: Docking results of quercetin, ellagic acid, ferulic acid, pthalic acid, ajugalactone, 20-hydroxyecdysone,camphene, elemol, α humulene, β mycrene, caffeic acid, chlorogenic acid, pcoumaric acid and transcinnamic acid with AChE receptor.

S.No	**Ligands**	**Binding Score (kJ/m-1)**	**Cavity size**	**Grid Map**	**HBA**
8	quercetin	-10	900	-42	7
9	ellagic acid	-9.3	1506	-54	8
10	ferulic acid	-7	1506	-54	3
11	pthalic acid	-6.9	1506	-54	2
12	ajugalactone	-8	1506	-54	8
13	20-hydroxy-ecdysone	-8.4	1208	-50	7
14	camphene	-6.5	900	-42	0
15	elemol	-7.1	900	-42	1
16	α humulene	-6.7	1506	-54	0
17	β mycrene	-6.2	900	-42	0
18	caffeicAcid	-7.4	1506	-54	3
19	chlorogenicAcid	-9.7	1506	-54	8
20	pcoumaricAcid	-6.9	1506	-54	2
21	transinamicAcid	-6.7	1506	-54	1

Continue TABLE 4.10: Docking results of quercetin, ellagic acid, ferulic acid, pthalic acid, ajugalactone, 20-hydroxyecdysone,camphene, elemol, α humulene, β mycrene, caffeic acid, chlorogenic acid, pcoumaric acid and transcinnamic acid with AChE receptor.

S.No	**Ligands**	**HBD**	**LogP**	**M.W (g/mol)**	**Rotatable Bond**
8	quercetin	5	1.988	302.23	1
9	ellagic acid	4	1.31	302.19	0
10	ferulic acid	2	1.498	194.18	3
11	pthalic acid	2	1.083	166.13	2
12	ajugalactone	4	2.163	516.6	3
13	20-hydroxy-ecdysone	6	1.854	480.6	5
14	camphene	0	2.998	136.23	0
15	elemol	1	3.942	222.37	3
16	α humulene	0	5.035	204.35	0
17	β mycrene	0	3.475	136.23	4
18	caffeicAcid	3	1.195	180.16	2
19	chlorgnicAcid	6	-0.645	354.31	4
20	pcoumaricAcid	2	1.49	164.16	2
21	transinmicAcid	1	1.784	148.16	2

Table 4.11 to 4.12 shows the interaction of ligands with BChE receptor. Among the selected ligands, ajugalactone show the highest binding score (-11.8 kcal/mol) followed by 20-hydroxyecdysone (-10.4 kcal/mol). Hydroquinone has the lowest binding score of -5.3 kcal/ mol.

TABLE 4.11: Docking results of hydroquinone, resorcinol, pyrocatechol, β sitosterols, ceryl alcohol, cerotic acid and palmitic acid with BChE receptor.

S.No	**Ligands**	**Binding Score (kJ/m-1)**	**Cavity size**	**Grid Map**	**HBA**
1	hydroquinone	-5.3	872	40	2
2	resorcinol	-5.4	872	40	2
3	pyrocatechol	-6.8	872	40	2
4	β sitosterols	-9.1	872	40	1
5	ceryl alcohol	-6.1	872	40	1
6	cerotic acid	-6.5	872	40	1
7	palmitic acid	-5.6	872	40	1

Continue TABLE 4.11: Docking results of hydroquinone, resorcinol, pyrocatechol, β sitosterols, ceryl alcohol, cerotic acid and palmitic acid with BChE receptor.

S.No	**Ligands**	**HBD**	**LogP**	**M.W (g/mol)**	**Rotatable Bond**
1	hydroquinone	2	1.097	110.11	0
2	resorcinol	2	1.097	110.11	0
3	pyrocatechol	2	3.344	194.27	2
4	β sitosterols	1	8.024	414.7	6
5	ceryl alcohol	1	9.361	382.7	24
6	cerotic acid	1	9.453	396.7	24
7	palmitic acid	1	5.552	256.42	14

TABLE 4.12: Docking results of quercetin, ellagic acid, ferulic acid, pthalic acid, ajugalactone, 20-hydroxyecdysone,camphene, elemol, α humulene, β mycrene, caffeic acid, chlorogenic acid, pcoumaric acid and transcinnamic acid with BChE receptor

S.No	**Ligands**	**Binding Score (kJ/m-1)**	**Cavity size**	**Grid Map**	**HBA**
8	quercetin	-9.9	872	40	7
9	ellagic acid	-10	872	40	8
10	ferulic acid	-7	872	40	3
11	pthalic acid	-6.7	872	40	2
12	ajugalactone	-11.8	872	40	8
13	20-hydroxy-ecdysone	-10.4	872	40	7
14	camphene	-5.6	872	40	0
15	elemol	-6.9	872	40	1
16	α humulene	-7.8	872	40	0
17	β mycrene	-5.2	872	40	0
18	caffeicAcid	-6.9	872	40	3
19	chlorogenicAcid	-8.9	872	40	8
20	pcoumaricAcid	-6.7	872	40	2
21	transinamicAcid	-6.5	872	40	1

Continue TABLE 4.12: Docking results of quercetin, ellagic acid, ferulic acid, pthalic acid, ajugalactone, 20-hydroxyecdysone,camphene, elemol, α humulene, β mycrene, caffeic acid, chlorogenic acid, pcoumaric acid and transcinnamic acid with BChE receptor

S.No	**Ligands**	**HBD**	**LogP**	**M.W (g/mol)**	**Rotatable Bond**
8	quercetin	5	1.988	302.23	1
9	ellagic acid	4	1.31	302.19	0
10	ferulic acid	2	1.498	194.18	3
11	pthalic acid	2	1.083	166.13	2
12	ajugalactone	4	2.163	516.6	3
13	20-hydroxy-ecdysone	6	1.854	480.6	5
14	camphene	0	2.998	136.23	0
15	elemol	1	3.942	222.37	3
16	α humulene	0	5.035	204.35	0
17	β mycrene	0	3.475	136.23	4
18	caffeicAcid	3	1.195	180.16	2
19	chlorgnicAcid	6	-0.645	354.31	4
20	pcoumaricAcid	2	1.49	164.16	2
21	transinmicAcid	1	1.784	148.16	2

4.5 Interaction of Ligands and Targeted Protein

Docking results generated through CB dock were analyzed using LigPlot and PyMol. Ligplot+ (version v.1.4.5) is used to predict the interaction between the ligands and the receptor proteins.

Ligplot+ graphical system automatically generates the 2D pictures of the interaction from its 3d coordinates [66].

The hydrophobic interactions and hydrogen-bond interaction pattern between the ligands and chains of proteins are shown as 2D diagrams in Figure 4.7- 4.48.

4.5.1 Interactions of Ligands with Acetylcholinestrase Receptor

Figure 4.7- 4.9 shows the interactions of ligands with best binding score with Target protein AChE. These figures were generated in 2 dimensions through Ligplot+. These 2 dimensional figures indicate the hydrogen bond and hydrophobic interactions of ligands with main chain or side chains of the protein. These hydrogen bond and hydrophobic interactions are listed in Table 4.13.

Figure 4.7 shows that hydroquinone forms three hydrogen bonds with Tyr123, Phe295 and Tyr337 and gives 4 hydrophobic interactions as mentioned in Table 4.8. As shown in Figure 4.7 the ligand, β sitosterol gives only one hydrogen bond but forms hydrophobic interactions with 3 residues. Ceryl alcohol, cerotic acid and palmitic acid give hydrophobic interactions with 13 residues as shown in Figure.

As indicated in Figure 4.8 ajugalactone only forms one hydrogen bond and no hydrophobic interactions. Camphene, α humulene and β mycrene do not form hydrogen bonds as its evident from their 2D structures they are without active oxygen atoms.

Maximum hydrogen bonds are shown by Caffeic acid, Pthalic acid and Ellagic acid as 5, 4 and 4 respectively. The ligand Caffeic acid made 5 hydrogen bonds and shows 5 hydrophobic interactions. The residues involved in hydrogen bonding are Tyr, Trp,Tyr and Glu as shown in Figure 4.9.

Its hydrophobic interactions are with Gly445, Tyr337, Ser135, Gly120 and Gly138. The ligand Pthalic acid made 4 hydrogen bonds and shows 4 hydrophobic interactions. The residues involved in hydrogen bonding are Ser,Arg,Phe and Tyr as shown in Figure. Its hydrophobic interactions are with Val294, Tyr124, Trp286 and Phe297.

The ligand Ellagic acid made 4 hydrogen bonds and shows 4 hydrophobic interactions. The residues involved in hydrogen bonding are Tyr, Phe and whereas it gives hydrophobic interactions with Asp74, Tyr341, Trp286, Tyr73 and His1394.

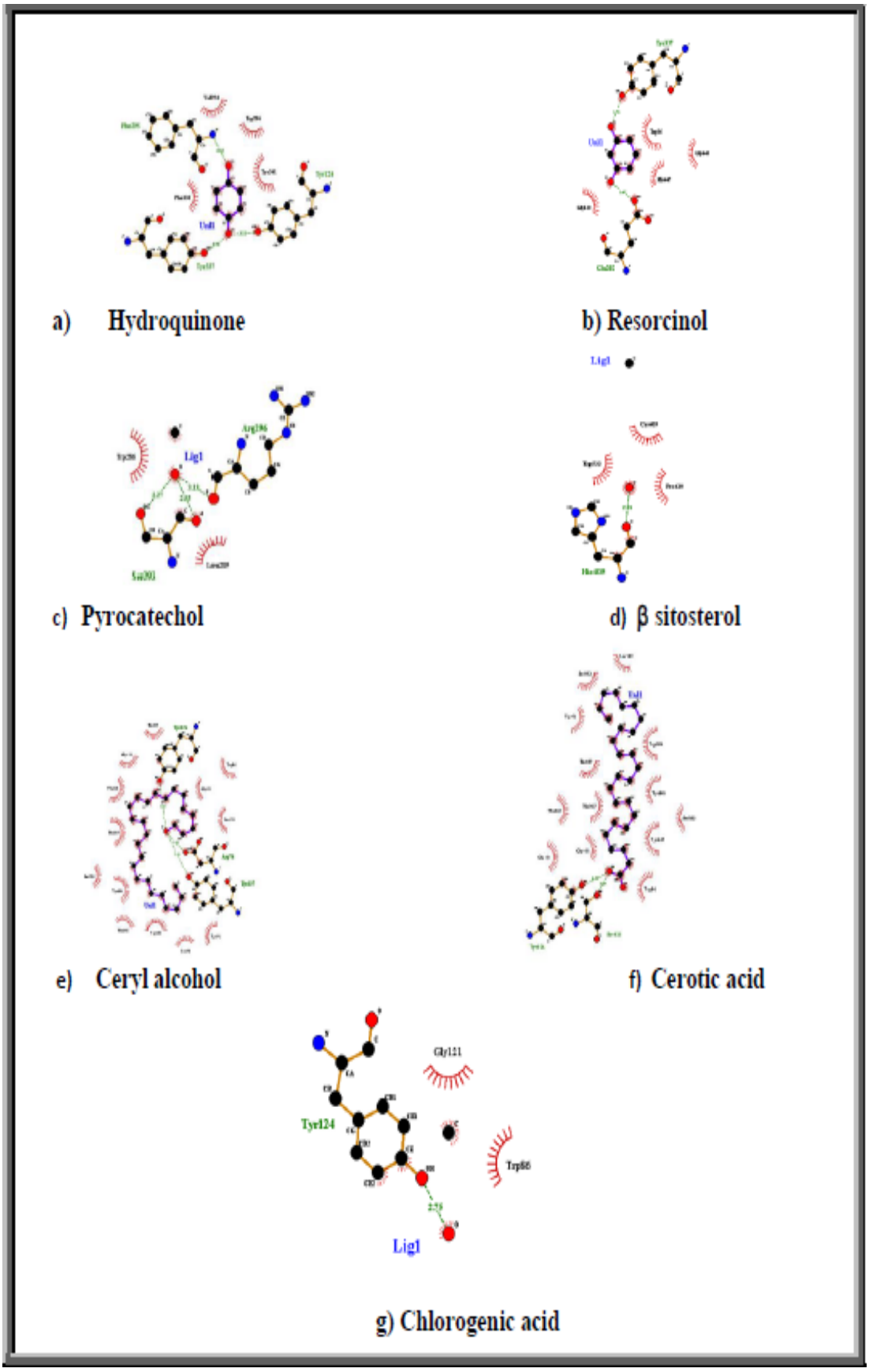


FIGURE 4.7: Interaction of Ligands with the receptor protein AChE. a) hydroquinone b) resorcinol c) pyrocatechol d) sitosterol e) ceryl alcohol f) cerotic acid g) chlorogenic acid

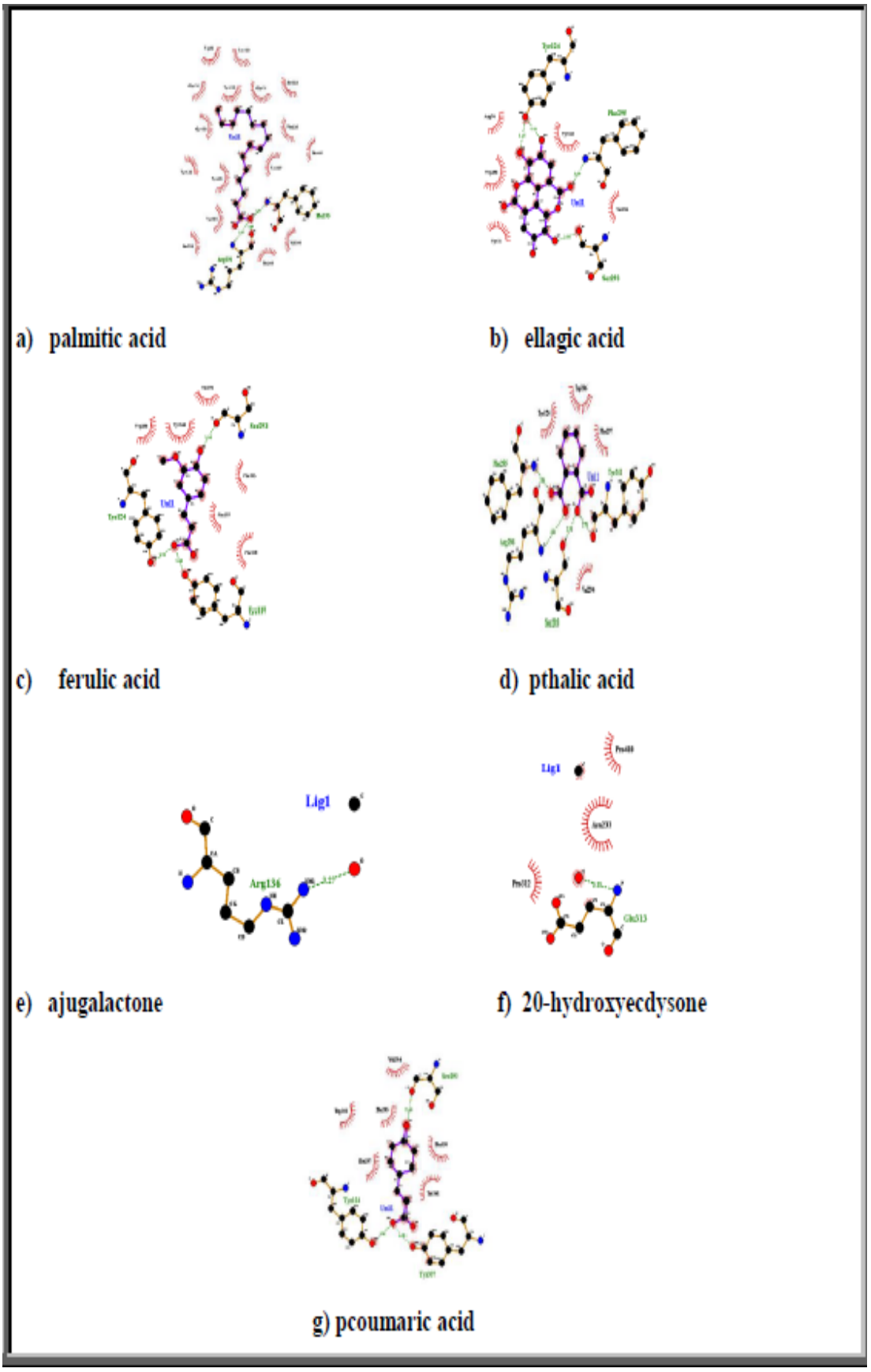


FIGURE 4.8: Interaction of Ligands with the receptor protein AChE. a) palmitic acid b)ellagic acid c) ferulic acid d) pthalic acid e) ajugalactone f) 20-hydroxyecdysone g) pcoumaric acid

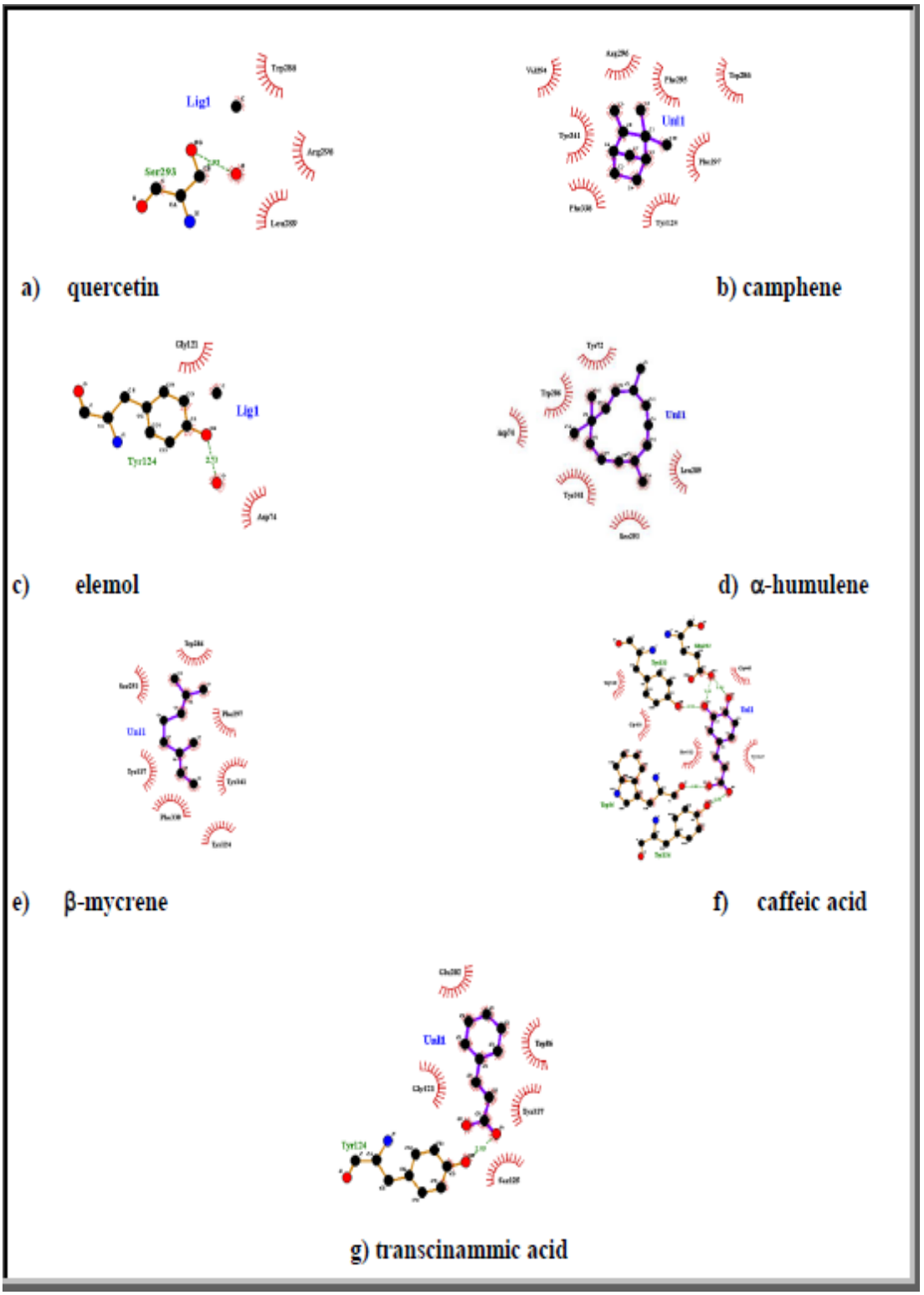


FIGURE 4.9: Interaction of Ligands with the receptor protein AChE. a) quercetin b) camphene c) elemol d) α humulene e) β mycrene f) caffeic acid g) transcinammic acid

The Table below shows the details of hydrogen bond and hydrophobic interactions of the selected ligand with the receptor protein AChE.

Table 4.13: Active Ligand showing Hydrogen and Hydrophobic Interactions with AChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
1	Hydroquinone	-5.5 & 3	Tyr337 Phe295 Tyr124	2.98 3.05 3.11	VAL294 Trp286 Tyr341 Phe338
2	Resorcinol	-5.7 & 2	Tyr337 Glu202	2.94 3.07	His447 Gly448 Gly120 Trp286
3	Pyrocatechol	-7.5 & 3	Ser293 Arg296 Ser293(B)	2.93 3.13 3.27	Leu289 Trp286
4	β sitosterols	-8.2 & 1	His405	2.91	Leu289 Trp532 Cys409 Pro410

Continue TABLE 4.19: Active Ligand showing Hydrogen and Hydrophobic Interactions with AChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding		Hydrophobic Bonding
			Amino Acids	Distance	
5	Ceryl alcohol	-6.7 & 3	Tyr124 Asp74 Tyr337	3.05 3.13 3.13	His447 Gly122 Phe338 Phe297 Ser293 Tyr341 Phe295 Trp286 Leu76 Tyr72 Ser125 Gly121 Trp86
6	Cerotic acid	-6.7 & 2	Tyr124 Ser135	2.99 3.63	Leu289 Ser293 Tyr72 His447

Continue TABLE 4.19: Active Ligand showing Hydrogen and Hydrophobic Interactions with AChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
					Phe338
					Phe297
					Gly121
					Gly122
					Trp337
					Trp86
					Tyr341
					Ser203
					Trp286
7	Palmitic acid	-6.9 & 3	Phe295	3.01	Leu130
			Arg296	3.01	Trp86
			Phe295(A)	2.97	Tyr124
					Gly121
					Gly126
					Ser203
					Gly120
					Tyr133

Continue TABLE 4.19: Active Ligand showing Hydrogen and Hydrophobic Interactions with AChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
					Tyr341
					Ser293
					Trp286
					Val294
					His447
					Phe297
					Phe338
					Tyr337
8	Ellagic acid	-9.3 & 4	Tyr124	3.15	Asp74
			Phe295	3.09	Tyr341
			Tyr124(B)	2.20	Trp286
			Ser293	2.85	Tyr73
					His1394
9	Ferulic acid	-7 & 3	Ser293	2.93	Val394
			Tyr124	2.92	Tyr341
			Tyr337	2.88	Trp286

Continue TABLE 4.19: Active Ligand showing Hydrogen and Hydrophobic Interactions with AChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
					Phe335
					Phe397
					Phe397
10	Pthalic acid	-6.9 & 4	Ser293	2.91	Val294
			Arg296	3.32	Tyr124
			Phe295	2.86	Trp286
			Tyr341	2.70	Phe297
11	Ajugalactone	-8 & 1	Arg136	3.27	0
12	20-hydroxyecdysone	-8.4 & 1	Glu313	3.21	Pro410
					Asn233
					Pro312
13	Quercetin	-10 & 1	Ser293	2.93	Leu289
					Arg296
					Trp286

Continue TABLE 4.19: Active Ligand showing Hydrogen and Hydrophobic Interactions with AChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding		Hydrophobic Bonding
			Amino Acids	Distance	
14	Camphene	-6.5 & 0	—	—	Val294 Tyr341 Phe338 Tyr124 Phe297 Phe295 Arg296 Trp286
15	Elemol	-7.1 & 1	Trp124	2.73	Asp74 Gly121
16	α humulene	-6.7 & 0	—	—	Leu289 Ser293 Tyr341 Asp74 Tyr72

Continue TABLE 4.19: Active Ligand showing Hydrogen and Hydrophobic Interactions with AChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding		Hydrophobic Bonding
			Amino Acids	Distance	
17	β mycrene	-6.2 & 0	—	—	Tyr124 Phe338 Tyr337 Tyr341 Phe297 Ser293 Trp286 Trp286
18	Caffeic acid	-7.4 & 5	Tyr124 Trp86 Tyr133 Glu202 Glu202(B)	3.86 3.03 2.99 2.99 2.96	Gly445 Tyr337 Ser135 Gly120 Gly138
19	Chlorogenic acid	-9.7 & 1	Tyr124	2.75	Gly121 Trp86

Continue TABLE 4.19: Active Ligand showing Hydrogen and Hydrophobic Interactions with AChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding		Hydrophobic Bonding
			Amino Acids	Distance	
20	Pcoumaric acid	-6.9 & 3	Tyr337 Tyr124 Ser293	3.95 3.95 3.03	Val294 Phe295 Phe297 Trp286 Tyr341 Phe338 Trp286
21	Transcinnamic acid	-6.7 & 1	Tyr124	2.83	Glu202 Gly121 Trp86 Tyr337 Ser125

4.5.2 Interactions of Ligands with Butyrylcholinestrase Receptor

The interactions of ligands with best binding score with Target protein BChE are shown in Figure 4.10- 4.12, while their hydrogen bond and hydrophobic interactions are listed in Table 4.14. The 2 dimensional picture of interaction of hydroquinone with target protein BChE is shown in Figure 4.10.

As shown in the figure, hydroquinone forms 2 hydrogen bonds and shows hydrophobic interactions with Ser198, Phe329, Gly116, Gly117, Trp231, Val288, Phe398. Ceryl alcohol shows maximum hydrophobic interactions as evident from the Figure 4.10.

It gives interactions with Ser198, Gly117, Trp231, Phe398, Gly116, Asp70, Ala328, Tyr440, Tyr332, Trp82, Thr120, Pro285, Ser287, Val288, Leu286 and Phe329.

As evident from the Figure 4.12 Camphene, α humulene, β mycrene form no hydrogen bonds as they are without active oxygen atoms. Maximum hydrogen bonds are shown by Caffeic acid, Pthalic acid and Ellagic acid as 5, 4 and 4 respectively.

The ligand caffeic acid forms 5 hydrogen bonds and 5 hydrophobic interactions. Caffeic acids forms bond with residues Tyr, Trp, Glu as shown in the Figure 4.12.

The ligand Transcinnamic acid forms 4 hydrogen bonds and 4 hydrophobic interactions. Transcinnamic acids forms bond with residues Trp440, Trp82, Trp430 and His as shown in the Figure 4.12.

The ligand Ellagic acid forms 4 hydrogen bonds and 5 hydrophobic interactions. Ellagic acids forms bond with residues Asp, Thr, Tyr, Glu. The ligand Transcinnamic acid forms 4 hydrogen bonds and 4 hydrophobic interactions. Transcinnamic acids forms bond with residues Trp440, Trp82, Trp430 and His as shown in the Figure 4.12. The ligand Ellagic acid forms 4 hydrogen bonds and 5 hydrophobic interactions. Ellagic acids forms bond with residues Asp, Thr, Tyr, Glu.

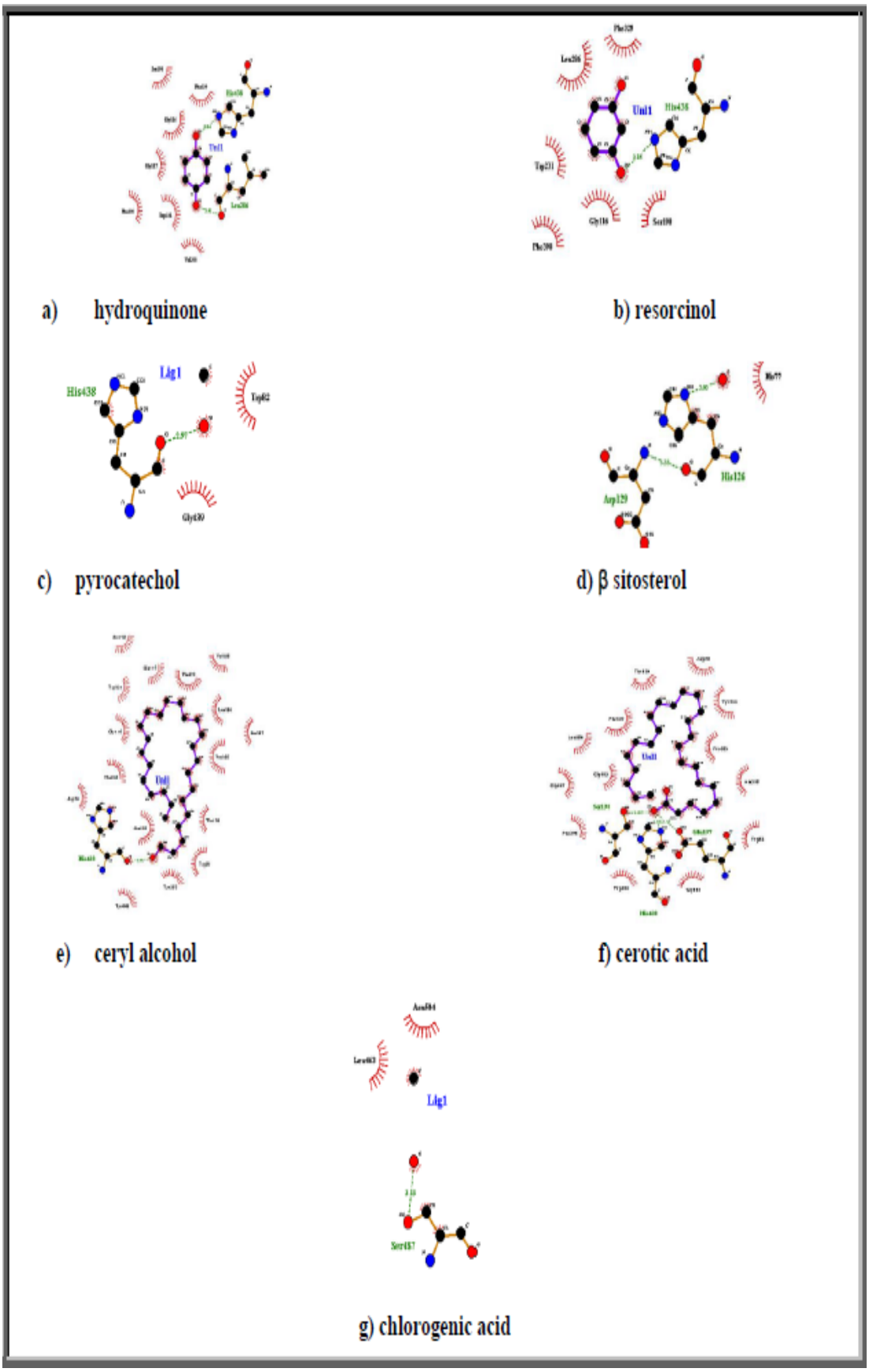


FIGURE 4.10: Interaction of Ligands with the receptor protein BChE. a) hydroquinone b) resorcinol c) pyrocatechol d) β sitosterol e) ceryl alcohol f) cerotic acid g) chlorogenic acid.

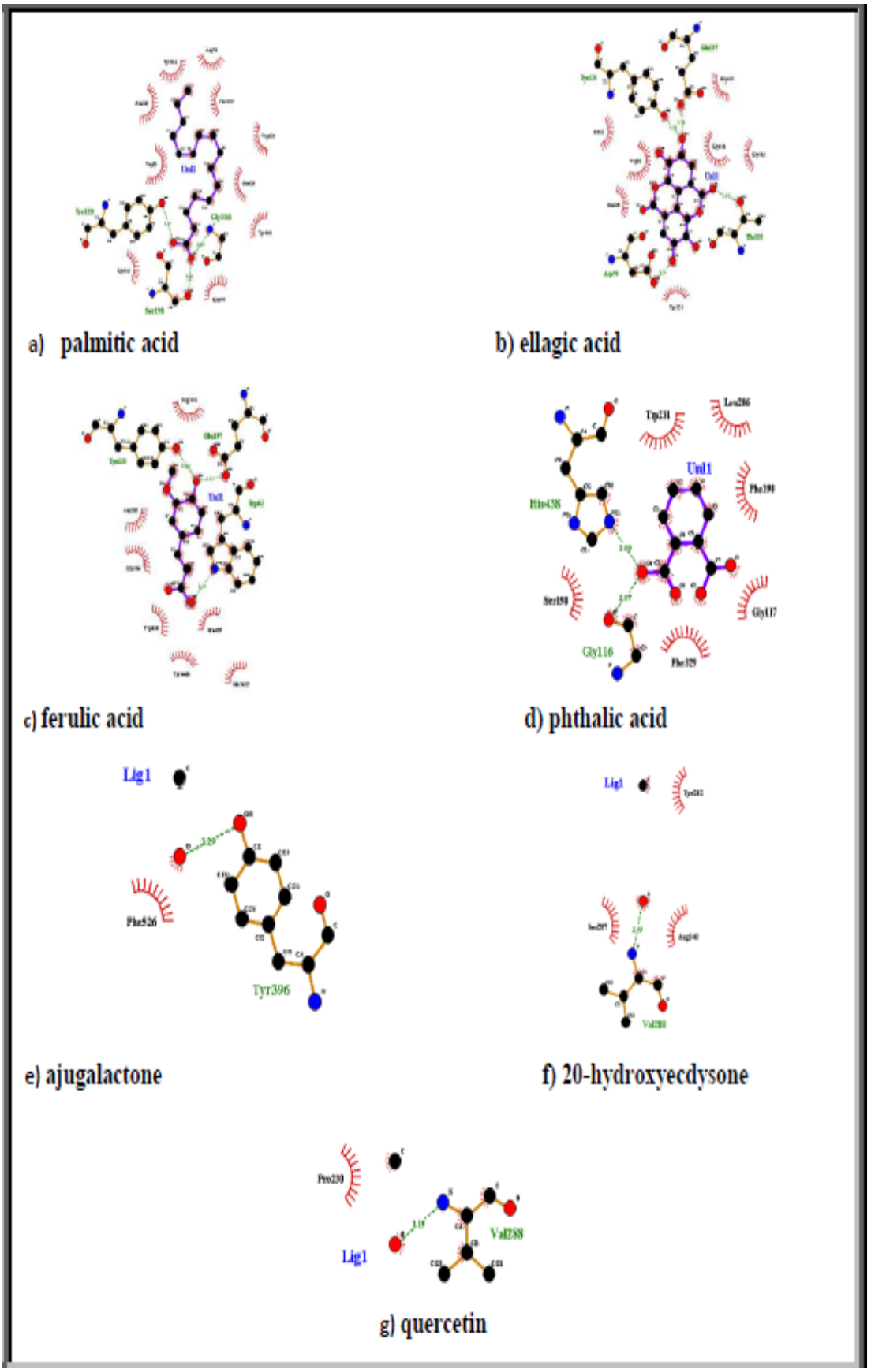


FIGURE 4.11: Interaction of Ligands with the receptor protein BChE. a) palmitic acid b)ellagic acid c) ferulic acid d) pthalic acid e) ajugalactone f) 20-hydroxyecdysone g) quercetin.

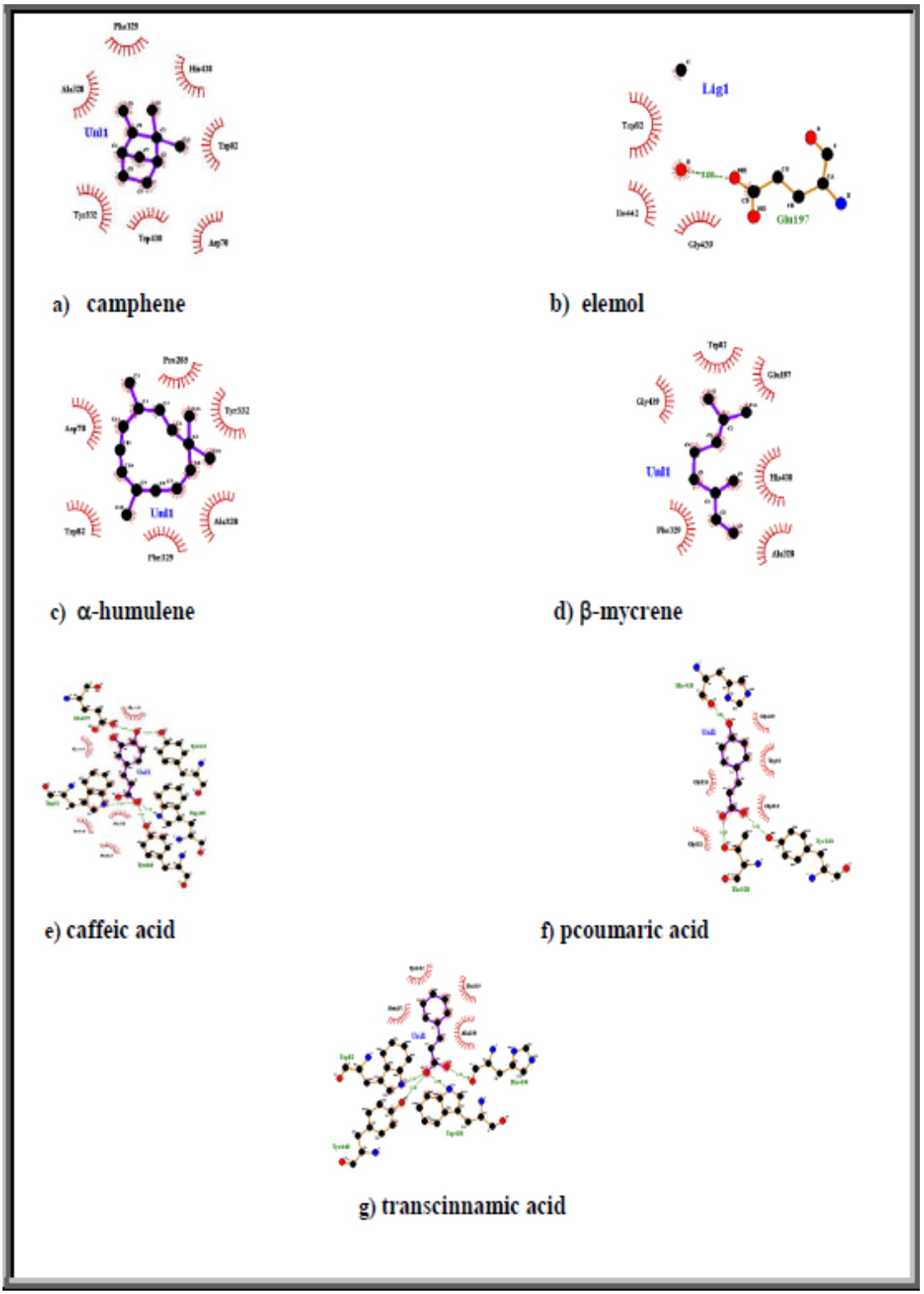


FIGURE 4.12: Interaction of Ligands with the receptor protein AChE. a) camphene b) elemol c) α humulene d) β mycrene e) caffeic acid f) pcoumaric acid g) transcinammic acid.

The Table below shows the details of hydrogen bond and hydrophobic interactions of the selected ligand with the receptor protein BChE.

Table 4.14: Active Ligand showing Hydrogen and Hydrophobic Interactions with BChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
1	Hydroquinone	-5.3 & 2	Leu286 His438	2.91 3.04	Val288 Trp231 Phe398 Gly117 Gly116 Phe329 Ser198
2	Resorcinol	-5.4 & 1	His438	3.15	Phe329 Leu286 Trp231 Gly116 Ser198
3	Pyrocatechol	-6.8 & 1	His438	2.97	Gly439 Trp82
4	β sitosterols	-9.1 & 2	Asp129 His126	3.35 3.03	His77

Continue TABLE 4.20: Active Ligand showing Hydrogen and Hydrophobic Interactions with BChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
5	Ceryl alcohol	-6.1 & 1	His438	3.01	Ser198 Gly117 Trp231 Phe398 Gly116 Asp70 Ala328 Tyr440 Tyr332 Trp82 Thr120 Pro285 Ser287 Val288 Leu286 Phe329
6	Cerotic acid	-6.5 & 3	Glu197	3.15	Thr130

Continue TABLE 4.20: Active Ligand showing Hydrogen and Hydrophobic Interactions with BChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
			Ser198	3.04	SePhe339
			His438	2.95	Leu356
					Gly115
					Gly117
					Phe395
					Trp331
					Gly116
					Trp53
					Ala325
					Pro255
					Tyr333
					Asp70
7	Palmitic acid	-5.6 & 3	Tyr128	3.27	Asp70
			Ser198	2.86	Tyr333
			Gly116	3.05	Ala325
					Trp53
					Gly115

Continue TABLE 4.20: Active Ligand showing Hydrogen and Hydrophobic Interactions with BChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
					Gly115
					Gla193
					Tyr440
					His435
					Trp430
					Phe339
8	Ellagic acid	-10 & 4	Asp70	2.71	Gly439
			Thr120	3.02	Gly115
			Tyr128	2.70	Gly116
			Glu197	2.74	Tyr333
					His435
					Ile443
					Trp53
9	Ferulic acid	-7 & 3	Tyr128	3.01	Gly115
			Glu197	2.91	Met437
			Trp82	3.31	Tyr440
					His435

Continue TABLE 4.20: Active Ligand showing Hydrogen and Hydrophobic Interactions with BChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
					Trp430
					Gly116
					Ala325
10	Pthalic acid	-6.7 & 2	His438	3.09	Ser198
			Gly116	3.07	Phe329
					Gly117
					Trp231
					Phe398
					Leu286
11	Ajugalactone	-11.8 & 1	Tyr396	3.29	Phe526
12	20-hydroxyecdsone	-10.4 & 1	Val288	2.99	Arg242
					Ser287
					Tyr282
13	Quercetin	-9.9 & 1	Val288	3.19	Pro230

Continue TABLE 4.20: Active Ligand showing Hydrogen and Hydrophobic Interactions with BChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding		Hydrophobic Bonding
			Amino Acids	Distance	
14	Camphene	-5.6 & 0	—	—	Phe329 Ala328 Tyr332 Trp430 Asp70 Trp82 His438
15	Elemol	-6.9 & 1	Glu197	3.08	Trp82 Ile442 Gly439
16	α humulene	-7.8 & 0	—	—	Asp70 Pro285 Tyr332 Ala328 Phe329 Trp82

Continue TABLE 4.20: Active Ligand showing Hydrogen and Hydrophobic Interactions with BChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding		Hydrophobic Bonding
			Amino Acids	Distance	
17	β mycrene	-5.8 & 0	—	—	Gly439 Trp82 Glu197 His438 Ala328 Phe329
18	Caffeic acid	-6.9 & 5	Tyr440 Trp430 Tyr128 Glu197 Trp82	3.30 3.18 2.9 1.95 3.23	Gly115 Gly116 Ala328 His438 Met137
19	Chlorogenic acid	-8.9 & 1	Ser487	3.13	Asn504 Leu463
20	Pcoumaric acid	-6.7 & 3	Tyr128	3.15	Gly439 Trp82

Continue TABLE 4.20: Active Ligand showing Hydrogen and Hydrophobic Interactions with BChE.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
			His438	3.54	Gly115 Gly116 Gly121
21	Transcinamic acid	-6.5 & 4	Trp440 Trp82 Trp430 His438	3.97 3.95 3.00 3.13	Tyr332 Met437 Ala328 Phe329

4.6 ADME Properties of Ligands

Lipinski rule of five is the initial step for evaluating drug oral bioavailability and artificial accessibility. Second step in drug screening is assessment of ADME properties of Ligands performed by inserting SMILIES in an online tool PkCSM.

4.6.1 Pharmacodynamics

Pharmacodynamics deals with the study of biochemical and physiological effects of drugs on the body. It is a broader term used in pharmacology [67].

4.6.2 Pharmacokinetics

Pharmacokinetics is another term used in pharmacology which deals with the study of drug affects in a living organism, the reaction of the body in response to induced drug. It also studies drugs absorption, distribution, metabolism and excretion properties [68].

4.6.3 Absorption

Absorption is one of ADME properties which indicate the absorption of orally administered drugs. It includes water solubility, $CaCO_2$ solubility, Intestinal absorption, skin permeability, P-glycoprotein substrate and P-glycoprotein I and II.

Water solubility of ligands gives values in log mol /L. This shows the solubility of a compound in water at 25^oC; hence water soluble drugs shown more solubility than lipid soluble drugs.

$CaCO_2$ solubility predicts the logarithm of apparent permeability coefficient. A compound has a high permeability if its value > 0.90 (log Papp in 10^-6 cm/s). Intestinal absorption indicates the value of the compound absorbed in the small intestine of a human. Poor absorbed values are less than 30%.

Skin permeability in Log p value indicates the permeability of a compound in skin. Compounds with values $>$ - 2.5 have low permeability. This model is important for transdermal drugs. P-glycoprotein substrate is an ABC transporter that extrudes toxins and other chemicals from entering cells by acting as a biological barrier. P-glycoprotein I and II predicts if a compound is inhibitor or not [69]. Table 4.15 shows the absorption properties of selected ligands taken through PkCSM.

Hydroquinone shows less solubility of water whereas CaCO2 solubility is in normal range. As evident from the table, hydroquinone shows intestinal absorption of 86.86%. Water solubility of resorcinol is low and does not act as a substrate of P-glycoprotein. It also has low permeability value in skin. The table indicates that compounds pyrocatechol and β sitosterol are not P-glycoprotein substrates but β sitosterols shows high absorption in humans and is also an inhibitor of P-glycoprotein I and II.

Ceryl alcohol, quercetin, cerotic and palmitic acid have low water solubility. The log Papp value of CaCO2 solubility is in normal range in these ligands except quercetin that shows low solubility. As mentioned in the table, ceryl alcohol, cerotic acid, palmitic acid and quercetin have low penetration in skin and are not P-glycoprotein substrates except Quercetin. Ceryl alcohol and cerotic acid are also the inhibitor of P-glycoprotein.

Ellagic acid, ferulic acid, pthalic acid, ajugalactone and 20-hydroxyecdysone also have low water solubility except ajugalactone. The values of CaCo2 solubility of all these ligands are also within normal range. All compounds can easily absorb in Intestine but show low skin penetration values. Ferulic shows highest intestinal absorption as indicated in the table.

The table shows that ellagic acid, ajugalactone and 20-hydroxyecdysone are P-glycoprotein substrates whereas ajugalactone is also an inhibitor of P-glycoprotein I. Camphene, elemol, β humulene and β mycrene have high solubility of CaCO2. All the compounds show good intestinal absorption and have low permeability of skin. α humulene is predicted as a P-glycoprotein substrate as shown in Table 4.15.

Caffeic acid, chlorogenic acid, pcoumaric acid and transcinnamic acid as mentioned in the table show less water solubility but CaCO2 solubility is within the normal range except for caffeic and chlorogenic acid. Transcinnamic acid shows highest intestinal absorption of about 94%, whereas chlorogenic acid has the lowest intestinal absorption. Skin permeability in terms of log Kp is low for these compounds. As indicated in the table chlorogenic acid is a substrate of P-glycoprotein.

TABLE 4.15: Absorption Properties of selected ligands.

Sr.no	Ligands	Water Solubility (mol/L)	CaCO2 Solubility (cm/S)	Intestinal Absorption (human)	Skin permeability (log Kp)	P-glyco-protein substrate	P-glyco-protein I inhibitor	P-glyco-protein IIinhibitor
1	Hydro-quinone	-0.762	1.679	86.86%	-2.618	No	No	No
2	Resor-cinol	-0.762	1.682	86.86%	-2.618	No	No	No
3	Pyrocat-echol	-2.737	1.635	89.58%	-2.481	No	No	No
4	β sito-sterol	-6.773	1.201	94.46%	-2.783	No	Yes	Yes
5	Quercetin	-2.925	-0.229	77.21%	-2.735	Yes	No	No
6	Ceryl-alcohol	-7.396	1.088	86.37%	-2.758	No	No	Yes
7	Cerotic-acid	-5.676	1.054	88.57%	-2.735	No	No	Yes
8	Palmitic-acid	-5.562	1.558	92.00%	-2.717	No	No	No
9	Ellagic-acid	-3.181	0.335	86.68%	-2.735	Yes	No	No
10	Ferulic acid	-2.817	0.176	93.69%	-2.72	No	No	No

Continue Table 4.15: Absorption Properties of selected ligands.

Sr.no	Ligands	Water Solubility (mol/L)	CaCO2 Solubility (cm/S)	Intestinal Absorption (human)	Skin permeability (log Kp)	P-glyco-protein substrate	P-glyco-protein I inhibitor	P-glyco-protein IIinhibitor
11	Pthalic-acid	-2.668	0.641	75.61%	-2.735	No	No	No
12	Ajugal-actone	-4.712	0.425	77.54%	-3.283	Yes	Yes	No
13	20-hydroxy-ecdys	-3.885	0.296	64.54%	-3.229 l	Yes	No	No
14	Camphene	-4.34	1.387	94.15%	-1.435	No	No	No
15	Elemol	-4.649	1.517	93.49%	-1.58	No	No	No
16	α humulene	-5.191	1.421	94.68%	-1.739 l	Yes	No	No
17	β mycrene	-4.497	1.4	94.70%	-1.043l	No	No	No
18	Caffeic acid	-2.33	0.634	69.41%	-2.722	No	No	No
19	Chlorogenic-acid	-2.449	-0.84	36.38%	-2.735	Yes	No	No
20	Pcoumaric acid	-2.378	1.21	93.49%	-2.715	No	No	No
21	Transcinnamic acid	-2.608	1.717	94.83%	-2.695	No	No	No

From the information gathered through PkCSM absorption running several compounds can be screened as a step behind from other ligands. Caffeic acid, chlorogenic acid, elagic acid, ferulic acid, ecdysteroids, pthalic acid, and Quercetin have low $CaCO_2$ solubility. α humulene, elagic acid; ajugalactone and 20-hydroxyecdysone are P-glycoprotein substrates. So these compound show less potential to be selected as lead compounds.

4.6.4 Distribution

Distribution in pharmacology deals with the spread of drugs throughout the body of living organism. The volume of distribution in humans (VDss), Fraction unbound in humans (Fu), Blood Brain Barrier (BBB), central nervous system permeability (CNS permeability), are four ADME properties [70]. The VDss expresses as L/Kg predicts the total dose of drug required to distribute uniformly throughout the blood plasma. If the value exceeds 2.81 L/kg then the drug is distributed more to the tissues than plasma however the VDss will be low if the value is below 0.71 L/kg. Compounds with more Fu values are more effective because as the drugs bind more to the serum proteins they will have less efficiency to diffuse to cellular membranes. Blood brain barrier is an important parameter as it protects the brain from exogenous compounds. Compounds with log BB $>$ 0.3 can easily cross the BBB barrier hence been effective and those compounds show poor distribution which have log BB$<$-1. Log PS is the product of surface area and Blood brain permeability. Compounds with a value of logPS$>$-2 penetrate the CNS whereas value log PS $<$-3 does not penetrate the CNS [71]. Table 4.16 shows the distributive properties of selected ligands. Hydroquinone, resorcinol, pyrocatechol and sitosterols have low values of VDss. Hydroquinone shows highest value of Fu among all these compounds. sitosterols can permeate the blood brain barrier but other compounds show poor permeability. Pyrocatechol and sitosterols have values less than -2 so they show poor penetration in CNS.

Ceryl alcohol, cerotic acid, palmitic acid and quercetin also have low values of VDss except quercetin that shows the VDss value as 1.55 L/kg. The range of

fraction unbounds is acceptable. As mentioned in the table only ceryl alcohol has blood brain permeability. All these compounds are not permeable in CNS except quercetin. Ellagic acid, ferulic acid, ecdysteroids , pthalic acid, ajugalactone and 20-hydroxyecdysone, as mentioned in the Table 4.16 show VDss values less than 0.45. The values of fraction unbound are in normal range. Pthalic acid shows low BBB permeability value as indicated in the table. All these compounds are permeable in CNS. β mycrene that shows a low value of VDss as 0.363 L/kg. Fraction unbound value of camphene, elemol, β mycrene and α humulene are in acceptable range. BBB permeability of all these compounds is high which indicates these can be effective. Elemol and α humulene have high CNS permeability value while the rest show less penetration. Caffeic acid, chlorogenic acid, pcoumaric acid and transcinnamic acid show VDss value high than 0.45. The fraction unbound values are within the range. The ligand transcinnamic acid can show penetration in brain tissue while the rest compounds have poor penetration. However, it has low penetration in CNS.

TABLE 4.16: Distributive properties of selected ligands.

S.No	**Ligands**	**VDss (human) (L/kg)**	**Fraction unbound (human)**	**BBB permeability**	**CNS permeability**
1	Hydroquinone	-0.022L/Kg	0.616Fu	-0.318log BB	-2.076log PS
2	Resorcinol	-0.022L/Kg	0.62Fu	-0.318log BB	-2.076log PS
3	Pyrocatechol	0.541L/Kg	0.173Fu	0.061log BB	-1.597log PS
4	β sitosterols	0.193L/Kg	0Fu	0.781log BB	-1.705log PS
5	Ceryl alcohol	0.144L/Kg	0.043Fu	0.987log BB	-1.192log PS
6	Cerotic acid	-0.637L/Kg	0.033Fu	-0.532log BB	-1.27log PS
7	Palmitic acid	-0.543L/Kg	0.101Fu	-0.111log BB	-1.816log PS
8	Quercetin	1.559L/Kg	0.206Fu	-1.098log BB	-3.065log PS
9	Ellagic acid	0.375L/Kg	0.083Fu	-1.272log BB	-3.533log PS
10	Ferulic acid	-1.367L/Kg	0.343Fu	-0.239log BB	-2.612log PS
11	Pthalic acid	-1.775L/Kg	0.497Fu	-0.038log BB	-2.891log PS

Continue Table 4.16: Distributive properties of selected ligands.

S.No	Ligands	VDss (human) (L/kg)	Fraction unbound (human)	BBB permeability	CNS permeability
12	Ajugalactone	-0.284L/Kg	0.303Fu	-0.898log BB	-3.476log PS
13	20-hydroxy-ecdysone	-0.547L/Kg	0.395Fu	-1.071log BB	-3.986log PS
14	Camphene	0.547L/Kg	0.354Fu	0.787log BB	-1.71log PS
15	Elemol	0.407L/Kg	0.245Fu	0.625log BB	-2.151log PS
16	α-humulene	0.505L/Kg	0.347Fu	0.663log BB	-2.555log PS
17	β-mycrene	0.363L/Kg	0.39Fu	0.781log BB	-1.902log PS
18	Caffeic acid	-1.098L/Kg	0.529Fu	-0.647log BB	-2.608log PS
19	Chlorogenic acid	0.581L/Kg	0.658Fu	-1.407log BB	-3.856log PS
20	Pcoumaric acid	-1.151L/Kg	0.428Fu	-0.225log BB	-2.418log PS
21	Transcinamic acid	-1.051L/Kg	0.38Fu	0.446log BB	-1.834log PS

4.6.5 Metabolism

Detoxification in liver is done by an enzyme Cytochrome P450. It releases xenobiotics when reacts with toxins. Usually drugs get deactivated in response to this enzyme but some remain active. Inhibitors of this enzyme are not to be used as they affect the metabolism of the drug [72]. Similarly CYP2D6 and CYP3A4 participate in drug metabolism. Inhibition of these cytochromes affects the pharmacokinetics of the available drugs. cerotic acid and palmitic acid work as a substrate of CYP3A4, whereas cerotic acid, ellagic and quercetin also are CYP1A2 substrate. As indicated in the table, all remaining ligands do not function as substrate or inhibitor of any of the isoform of enzyme, Cytochrome P450.

Table 4.17: Metabolic properties of selected ligands.

Sr.no	Ligands	CYP2D6 substrate	CYP3A4 substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
1	Hydroquinone	No	No	No	No	No	No	No
2	Resorcinol	No	No	No	No	No	No	No
3	Pyrocatechol	No	No	Yes	No	No	No	No
4	β sitosterol	No	Yes	No	No	No	No	No
5	Quercetin	No	No	Yes	No	No	No	No
6	Cerylalcohol	No	Yes	No	No	No	No	No
7	Cerotic acid	No	Yes	Yes	No	No	No	No
8	Palmitic acid	No	Yes	No	No	No	No	No
9	Ellagic acid	No	No	Yes	No	No	No	No
10	Ferulic acid	No	No	No	No	No	No	No

Continue Table 4.17: Metabolic properties of selected ligands.

Sr.no	Ligands	CYP2D6 substrate	CYP3A4 substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
11	Pthalic acid	No	No	No	No	No	No	No
12	Ajugalactone	No	No	No	No	No	No	No
13	20-hydroxyecdysone	No	No	No	No	No	No	No
14	Camphene	No	No	No	No	No	No	No
15	Elemol	No	No	No	No	No	No	No
16	α humulene	No	No	No	No	No	No	No
17	β mycrene	No	No	No	No	No	No	No
18	Caffeic acid	No	No	No	No	No	No	No
19	Chlorogenic acid	No	No	No	No	No	No	No
20	Pcoumaric acid	No	No	No	No	No	No	No
21	Transcinnamic acid	No	No	No	No	No	No	No

4.6.6 Excretion

Excretion of drugs is mainly performed by kidneys and liver. Lungs can also take part in excretion by eliminating gaseous or volatile substances. Sweat, saliva and tears can also excrete drugs. Renal OCT2 substrate is the transporter that clears out the drugs and other compounds. Renal clearance indicates the excretion values of drugs and Total clearance indicates hepatic clearance which means the drug is metabolized [73]. Excretory properties of ligands are listed in tables below. Table 4.13 shows the excretory properties of hydroquinone, resorcinol, pyrocatechol and sitosterols. Total clearance values of all these ligands are within normal range. As indicated in the table, all compounds show negative result as a substrate of renal OCT2. Ellagic acid, ferulic acid, pthalic acid, ajugalactone and 20-hydroxyecdysone as mentioned in Table show total clearance values of within the recommended range. As indicated in the table, all these compounds give negative result as a substrate of renal OCT2. Camphene, elemol, α humulene and β mycrene show normal range of Total clearance value. As mentioned in the table, all compounds show negative result as a substrate of renal OCT2. Caffeic acid, chlorogenic acid, pcoumaric acid and transcinnamic acid as mentioned in Table 4.18 show Total clearance values within the recommended range. As indicated in the table, all these compounds show negative result as a substrate of renal OCT2.

TABLE 4.18: Excretory properties of selected ligands.

S No	Ligands	Total Clearance	Renal OCT2
1	Hydroquinone	0.52 ml/Kg	No
2	Resorcinol	0.238 ml/Kg	No
3	Pyrocatechol	0.271 ml/Kg	No
4	β sitosterols	0.628 ml/Kg	No
5	Ceryl alcohol	2.104 ml/Kg	No
6	Cerotic acid	1.939 ml/Kg	No
7	Palmitic acid	1.763 ml/Kg	No
8	Quercetin	0.407 ml/Kg	No
9	Ellagic acid	0.537 ml/Kg	No

10	Ferulic acid	0.623 ml/Kg	No
11	Pthalic acid	0.682 ml/Kg	No
12	Ajugalactone	0.469 ml/Kg	No
13	20-hydroxyecdysone	0.576 ml/Kg	No
14	Camphene	0.049 ml/Kg	No
15	Elemol	1.311 ml/Kg	No
16	α humulene	1.282 ml/Kg	No
17	β mycrene	0.438 ml/Kg	No
18	Caffeic acid	0.508 ml/Kg	No
19	Chlorogenic acid	0.307 ml/Kg	No
20	Pcoumaric acid	0.662 ml/Kg	No
21	Transcinnamic acid	0.781 ml/Kg	No

4.7 Lead Compound Identification

The physiochemical and pharmacokinetic properties of the ligands determine the destiny as drug or non-drug like compound. The first filter for this identification is Lipinski rule of Five and second screening is done through pharmacokinetic properties. Those compounds which do not follow more than 2 rules are not considered as drug like. Ajugalactone shows more molecular weight than 500, but follows other two factors so it is still acceptable. The Log P value of β sitosterol, ceryl alcohol, cerotic acid and palmitic acid were more than 5 but they were still passed to next stage. So, in first screening there were no knockouts and all compounds were passed for next stage. The next knockout step is pharmacokinetic screening. In this screening, β sitosterol and ceryl alcohol both were knocked out as they are hERG II inhibitors. Cerotic acid had also been knocked out because it is hepatotoxic and can damage the liver [74]. At the end of this the compounds left were hydroquinone, resorcinol, pyrocatechol, palmitic acid, elagic acid, ferulic

acid , pthalic acid, ajugalactone ,20-hydroxyecdysone, quercetin, camphene, elemol, α humulene, β mycrene, caffeic acid, chlorogenic acid, pcoumaric acid and transcinnamic acid. Among all these QUERCETIN and ELLAGIC ACID were identified as top compounds for drug production. But as ellagic acid shows four hydrogen bonds and five hydrophobic interactions with both AChE and BChE wherease quercetin shows one hydrogen and three hydrophobic interactions with AChE and one hydrogen bond and one hydrophobic interaction with BChE, so ELLAGIC ACID was selected as leading compound of this research.

4.8 Drug Identification Against Alzheimer Disease

The U.S Food and Drug Administration (FDA) approved medications for treatment of Alzheimer disease. These work either by delaying the onset of the disease or by working as inhibitor molecules for Cholinestrase enzymes. One of the drugs that have been used in different countries around the world like UK, Brazil, India and Pakistan is Donepezil. Even though the drug still has some reserves but its use has greatly increased over the past years [75].

4.8.1 Donepezil

Donepezil sold under the name, Aricept is an oral medication which is used against the action of Cholinesterase enzymes. It is an FDA approved drugs used to relieve the symptoms of mild to severe Alzheimers. It prevents the breakdown of acetylcholine in brain. Improvements have been observed in cognition and behavior of patients suffering from Alzheimer disease. With that donepezil is also used as a glutamate regulator when it is taken with the combination of memantine [76]. Other than its effect on cholinesterases in the brain, donepezil also stimulates pathways of APP processing by releasing -secretase and also up-regulates the nicotinic receptors in cortical neurons hence increasing neuroprotection [77].

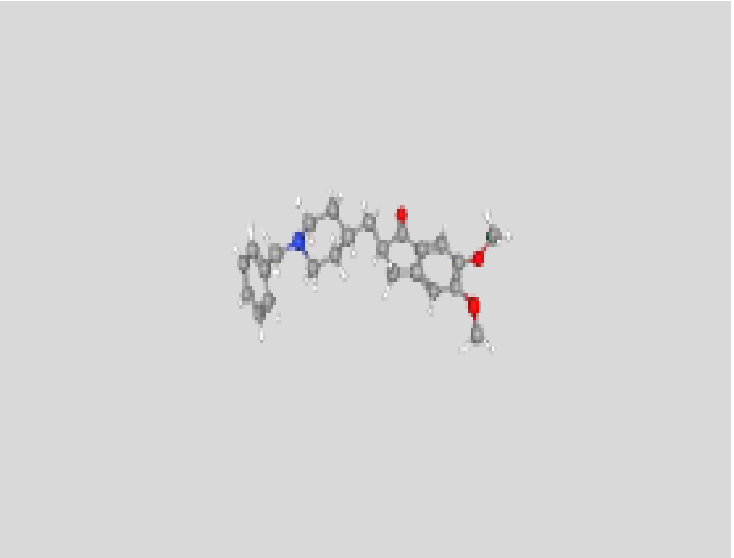


FIGURE 4.13: 3D structure of Donepezil from PubChem.

4.9 Drug ADMET Properties

The Drug ADMET properties are studied using the same software as above which is PkCSM. These values of toxicity, absorption, distribution, metabolism and excretion of reference drug are listed in Table 4.14. The toxicity value of the Drug indicates maximum tolerated dose of -0.217 whereas the drug also predicts itself as a hERG II inhibitor which means it can inhibit potassium channels. It shows hepatotoxicity which means it can cause liver injury. Donepezil predicts minnow toxicity as -2.011log Mm. Absorption Properties of Donepezil are mentioned in the Table 4.19.As evident from the table, it shows very less solubility in water and has 93.70% absorption capacity in small intestine of humans. Permeability of the drug in skin is low and shows a positive result as P-glycoprotein substrate and P-glycoprotein I/II inhibitor. This indicates that the standard drug has low oral absorption and a reduced ability to pump xenobiotic out of the cells. Distribution properties of reference drug indicate that donepezil shows high VDss value which means it is distributed more in tissue and less in plasma. Fu predicts the fraction unbound in plasma. Drugs which show more values are more effective. Standard drug shows a predicted value of 0 Fu. The third model BBB permeability is 0.157 log BB which means it's poorly permeable to the brain. CNS permeability expressed as log PS $>$ -3 is considered as poorly permeable while donepezil

shows the value of -1.464 log PS. Cytochrome P450 is a detoxification enzyme in liver and is involved in excretion of exogenous compounds. The main isoforms of this enzyme are CYP2D6 and CYP3A4. 1st and 2nd model of metabolic properties indicates that donepezil is metabolized by both isoforms. Model 3-5 indicate that donepezil is not an inhibitor of these isoforms whereas model 6 and 7 show donepezil as an inhibitor. The predicted values for excretion of drug are listed in Table 4.19. Hepatic and renal clearance are shown as Total clearance, expressed as log (CL tot). This value is 0.987 ml/kg. As it predicts renal OCT2 clearance as "YES" which means it interferes with the functioning of OCT2 in cells.

Table 4.19: Excretory properties of selected ligands.

S No	ADMET Properties	Model Name	Donepezil
01	**Toxicity**	AMES toxicity	No
		Max. tolerated dose (human)	-0.217 mg/kg
		hERG I inhibitor	No
		hERG II inhibitor	Yes
		Oral rat acute toxicity	2.753 mol/kg
		Oral rat chronic toxicity	0.991 mg/kg
		Hepatotoxicity	Yes
		Skin sensitization	No
		T. pyriformis toxicity	0.804 log ug/L
		Minnow toxicity	-2.011 log Mm.
02	**Absorption**	Water solubility	-4.648 mol/L
		CaCO2 solubility	1.273 cm/S
		Intestinal Absorption (human)	93.707%
		Skin permeability	-2.585 log Kp
		P-glycoprotein substrate	Yes
		P-glycoprotein I inhibitor	Yes
		P-glycoprotein II inhibitor	Yes
		-VDss (human)	1.266 L/kg
		Fraction unbound (human)	0 Fu

03	**Distribution**	BBB permeability	0.157 log BB
		CNS permeability	-1.464 log PS
04	**Metabolosim**	CYP2D6 substrate	Yes
		CYP3A4 substrate	Yes
		CYP1A2 inhibitor	No
		CYP2C19 inhibitor	No
		CYP2C9 inhibitor	No
		CYP2D6 inhibitor	Yes
		CYP3A4 inhibitor	Yes
05	**Excretion**	Total Clearance	0.987 ml/kg
		Renal OCT2 Substrate	Yes

4.10 Donepezil Mechanism of Action

Donepezil is a piperdine derivative and is a reversible cholinesterase inhibitor with a plasma life of about 70 hours. Acetylcholinestrase and Butyrylcholinestrase are the enzymes that hydrolyze the acetylcholine in the synaptic cleft. Donepezil binds to these inhibitor molecules and prevent the hydrolysis reaction thus increasing the amount of acetylcholine in the cerebral cortex and other areas of the brain and enhances the cholineragic transmission [78]. It is also used to neuroprotect the body by causing up regulation of nicotinic receptors in cortical neurons. It also obstructs the voltage induced sodium current while slowing down the potassium currents [79]. Moreover, donepezil also shows antagonism of the 1 receptor (Ki = 14.6 nM), and shows antiamnestic effects because of this action in animals. It also shows interaction with agents FK960 and FK962 for somatostatingic neurotransmission. While interacting with atacurium donepezil can also narrow down the magnitude of neuromuscular blockage. But these interactions can also lead to an increased risk of bradycardia [80]. For these reasons the administration of donepezil is linked with antiobiotic resistance as it can cause abnormal heart

rhythm that could lead to heart failure resulting in death. Figure 4.44 shows the mechanism of action of donepezil.

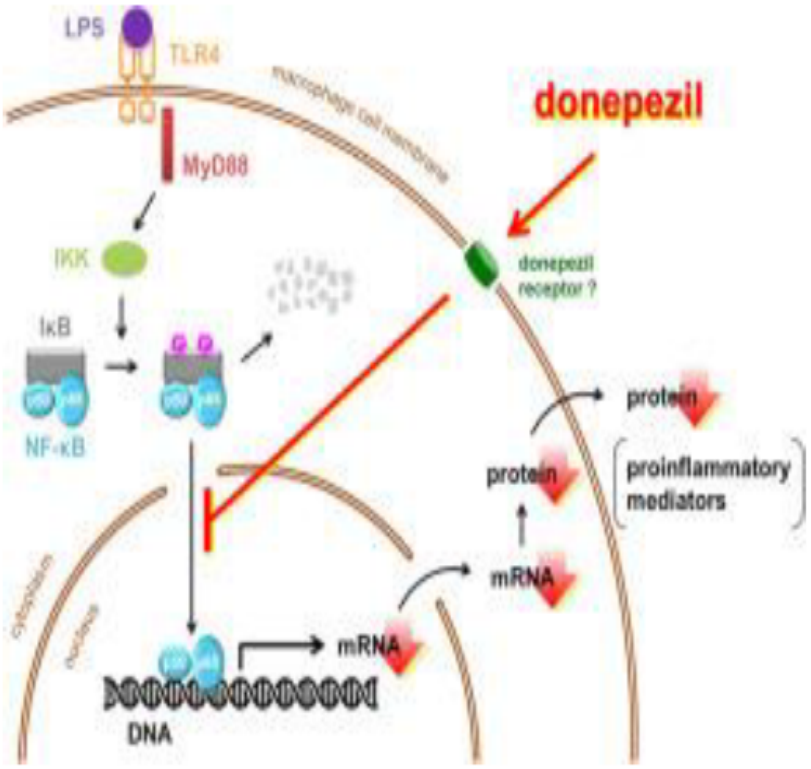


FIGURE 4.14: Mechanism of action of donepezil [81].

4.11 Donepezil Effects on the Body

Donepezil is anticholinestrase inhibitor used to prevent the hydrolysis of acetylcholine. The most common side effects that occur after the medication are that occur are nausea, vomiting, diarrhea, headache, weight loss, trouble sleeping, muscle cramps and dizziness [82].

Long term use of this drug could lead to allergic reactions that could lead to swelling of face/ tongue and infections. With that in rare case leads to irregular heartbeat.

4.12 Donepezil Docking

Docking was performed with donepezil as a ligand using online tool CB dock. AChE and BChE were used as drug target in this research work. Donepezil showed best binding score as -10 with AChE and -9.6 with BChE. Molecular docking interactions with both target protein are mentioned below in **Table 4.20**.

Table 4.20: Docking result of Donepezil with AChE and BChE.

S.No	Compound	Donepezil with AChE	Donepezil with BChE
1	Binding Score	-10	-9.6
2	HBD	0	0
3	HBA	4	0
4	logP	4.3611	4.3611
5	Molecular Weight g/mol	379.5 g/mol	379.5 g/mol
6	Rotatable Bonds	6	6
7	Grid Map	-54	40
8	Cavity Size	1506	872

4.13 Donepezil Comparison with Lead Compound

The standard drug and lead compound are compared and their physiochemical and pharmacokinetic properties are assessed for the bioavailability, efficacy, safety and drug likeliness. Both compounds pass the Lipinski rule criteria for drug-likeness. However, ellagic acid shows less molecular weight and Log P value (**Table 4.21**).

Table 4.21: Lipinski Rule Comparison.

S.No	Drug	logP Value	Molecular Weight	H-Bond Acceptor	H-Bond Donor
1	Donepezil	4.3611	379.5 g/mol	0	4
2	Ellagic acid	1.31	302.194 g/mol	4	8

4.14 ADMET Properties Comparison

Pharmacokinetic properties play a major role in selection of compounds as drug candidates. The ADMET properties comparison is performed to check adsorption, distribution, metabolism, excretion and toxicity. The comparison of ADMET properties of the standard drug, donepezil and leading compound, ellagic acid is listed in the Table 4.22. The most critical parameter of ADMET properties is Toxicity. The toxicity of compounds is checked on the basis of 9 models. As per Model 1 of AMES toxicity both drug and lead compound are non-mutagenic. Model 2 indicates as both compounds have value less than 0.477 log mg/kg/ day, so both have low value of tolerated dose. 3rd Model is hERG I and II inhibitors. Donepezil is hERG II inhibitor which means it can lead to QT syndrome but Ellagic acid is not an inhibitor. Relative toxicity is assessed from 4th model of oral rat acute toxicity. Model 5 of oral rat chronic toxicity shows the lowest value of dose that can cause adverse effects. Model 6 of hepatotoxicity shows that Donepezil is hepatotoxic and can damage the liver. Both the standard and lead compounds are not skin sensitive as per Model, so they can be used for dermal products. Model 8 shows T. pyriformis toxicity and value >-0.5 is considered toxic. According to which, donepezil is toxic (Table 4.22). Model 9 uses minnows to check toxicity and values below 0.5mm are considered toxic. Donepezil shows toxicity but Ellagic acid passes this toxicity test. From Table below, it is evident that water solubility of standard drug is less than the leading compound. The model of CaCO2 solubility predicts the absorption of oral drugs. Both compounds can get absorbed. Compounds showing less than 30% of intestinal absorption get poorly absorbed. Both the compounds pass this test. For transdermal drugs the skin permeability model is used, compounds showing values log Kp >-2.5 are considered low, both compounds pass this test. Both the compounds are P-glycoprotein substrate which is very important as P-glycoprotein is an ABC transporter and works as a biological barrier. The Table indicates that ellagic acid not an inhibitor of P-glycoprotein, whereas donepezil shows inhibition. The distribution properties of compounds can be detected through 4 models as shown in **Table 4.21**. 1st

model VDss gives the uniform distribution of blood plasma. Compounds that show values above 2.81L/kg indicate drug distribution more to tissues than plasma. Both the compared compounds show logical VDss values. Fu value of ellagic acid is more than donepezil which indicates that ellagic acid is more effective than standard drug in case of unbounded fraction in plasma. Drugs that show BBB permeability value > 0.3 log BB than those can easily cross the blood brain barrier and those that have value <-1 logBB can be poorly absorbed. Similarly, CNS model indicates that drugs with Log PS > -2 then the drug can penetrate the CNS while those with values <-3 cannot show permeability. Donepezil has low value so cannot penetrate the central nervous system. Metabolic Properties are based on isoforms of cytochrome P450 which are CYPD2D6, CYP3A4, CYP1A2, CYP2C19 and CYP2C9. Cytochrome P450 is present in liver and is utilized in oxidizing the xenobiotics so they can be eliminated from the body and works as detoxifying enzyme [83]. Table below indicates that donepezil is substrate of both CYPD2D6 and CYP3A4 whereas ellagic acid is not a substrate. Ellagic acid is inhibitor of CYP1A2, whereas donepezil is inhibitor of CYP2D6 and CYP3A4 as shown in Table below. Excretion properties consist of two model and their values are given in table below. Ellagic acid shows less total clearance than the standard drug. The **Table 4.22** indicates 2nd model of Renal OCT2 substrates. Being OCT2 substrates has harmful effects when related with inhibitors. Ellagic acid is not a substrate but donepezil acts as a substrate.

Table 4.22: Excretory properties of selected ligands.

S No	ADMET Properties	Model Name	Donepezil	Ellagic Acid
01	**Toxicity**	AMES toxicity	No	No
		Max. tolerated dose(human)	-0.217 mg/kg	-0.217 mg/kg
		hERG I inhibitor	No	No
		hERG II inhibitor	Yes	No
		Oral rat	2.753 mol/kg	2.399 mol/kg

		acute toxicity		
		Oral rat chronic toxicity	0.991 mg/kg	2.698 mg/kg
		Hepato toxicity	Yes	No
		Skin sensitization	No	No
		T. pyriformis toxicity	0.804 log ug/L	0.295 log ug/L
		Minnow toxicity	-2.011 log mM	2.11 log mM
02	**Absorption**	Water solubility	-4.648 mol/L	-3.181 mol/L
		CaCO2 solubility	1.273 cm/S	0.335 cm/S
		Intestinal Absorption (human)	93.707%	86.684%
		Skin permeability	-2.585 log Kp	-2.735 log kp
		P-glycoprotein substrate	Yes	Yes
		P-glycoprotein I Inhibitor	Yes	No
		P-glycoprotein II Inhibitor	Yes	No
		VDss (human)	1.266 L/kg	0.375 L/kg
		Fraction unbound	0 Fu	0.083 Fu

03	**Distribution**	(human) BBB permeability	0.157 log BB	-1.272 log BB
		CNS permeability	-1.464 log PS	-3.533 log PS
04	**Metabolosim**	CYP2D6 substrate	Yes	No
		CYP3A4 substrate	Yes	No
		CYP1A2 inhibitor	No	Yes
		CYP2C19 inhibitor	No	No
		CYP2C9 inhibitor	No	No
		CYP2D6 inhibitor	Yes	No
		CYP3A4 inhibitor	Yes	No
05	**Excretion**	Total Clearance	0.987 ml/kg	0.537 ml/kg
		Renal OCT2 Substract	Yes	No

4.15 Physiochemical Properties Comparison

Physiochemical properties show the basic and fundamental characteristics of the compounds [84]. These also work as primary screener for desirable properties. Donepezil is composed of 24 atoms of carbon, 29 hydrogen, 1 nitrogen and 3

oxygen whereas ellagic acid shows 14 carbons, 6 hydrogen and 8 oxygen. This shows that ellagic acid is a simple compound as compared to donepezil. The molecular weight of ellagic acid is 302.19 whereas donepezil has 379.5 and Log P value of donepezil is also higher than ellagic acid as shown in **Table 4.23**. Ellagic acid donates 4 more hydrogen atoms than donepezil that indicates its oxidation state. Donepezil accepts 4 hydrogen bonds whereas ellagic acid has 8. Higher than 10 rotatable bonds show decreased oral bioavailability. Donepezil shows 6 rotatable bonds whereas ellagic acid has zero.

TABLE 4.23: Physiochemical properties comparison

S.No	**Parameters**	**Donepezil**	**Ellagic acid**
1	Molecular formula	$C_{24}H_{29}NO_3$	$C_{14}H_6O_8$
2	HBD	0	4
2	HBA	4	8
4	log p	4.3611	1.31
5	Molecular Weight	379.5 g/mol	302.19 g/mol
6	Rotatable Bond	6	0

4.16 Docking Score Comparison

Both the standard drug and lead compounds were docked with receptor proteins AChE and BChE. The **Table 4.24** indicates the best pose docking scores. Ellagic acid shows a bonding score of -10 with BChE and -9.3 with AChE. whereas Donepezil shows -9.6 and -10 with BChE AChE, indicated in the table below.

TABLE 4.24: Distributive properties of selected ligands.

S.No	**Name**	**Docking score with AChE**	**Docking score with BChE**
1	Donepezil	-10	-9.6
2	Ellagic acid	-9.3	-10

4.17 Docking Analysis Comparison

4.17.1 Docking analysis comparison with AChE

The Docking results are visualized using LigPlot and analyzed on the basis of number of hydrogen bonds and hydrophobic interactions, number of steric interactions and number of interacting amino acids. The 2d diagrams generated through Ligplot by the interaction of donepezil and ellagic acid with AChE is shown in **Figure 4.14**.

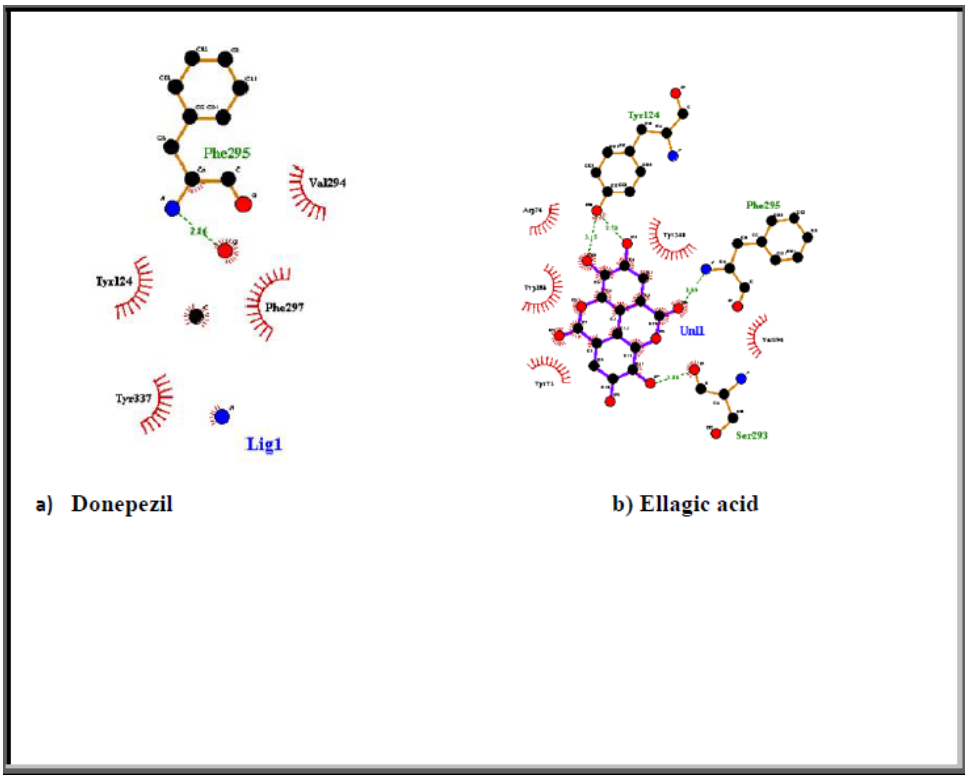


FIGURE 4.15: Interaction of Donepezil and Ellagic acid with receptor protein AChE.

The hydrogen bond and hydrophobic interaction details are shown in the **Table 4.25**. Presence of oxygen atoms is important as it's critical for H- bond formation with the target protein. Ellagic acid forms 4 hydrogen bonds with residues Tyr, Phe, and Ser, whereas donepezil forms only 1 hydrogen bond with Phe. Furthermore, hydrophobic interactions of ellagic acid are five in number with residues Asp74, Tyr341, Trp286, Tyr73 and His1394 whereas donepezil forms four interactions with Val294, Phe297, Tyr124 and Tyr337.

TABLE 4.25: Docking analysis Comparison with receptor protein AChE.

S.No	Compound	Binding Energy & No of HBs	Hydrogen Bonding		Hydrophobic Bonding
			Amino Acids	Distance	
1	Donepezil	-10 & 1	Phe295	2.86	VAL294 Phe297 Tyr124 Tyr337
2	Ellagic acid	-9.3 & 4	Tyr124 Phe295 Tyr124(B) Ser293	3.15 3.09 2.20 2.85	Asp74 Tyr341 Trp286 Tyr73 His1394

4.17.2 Docking Analysis Comparison with BChE

The 2d diagrams generated through Ligplot by the interaction of donepezil and ellagic acid with BChE is shown in Figure 4.15 and the properties are mentioned in Table 4.26.

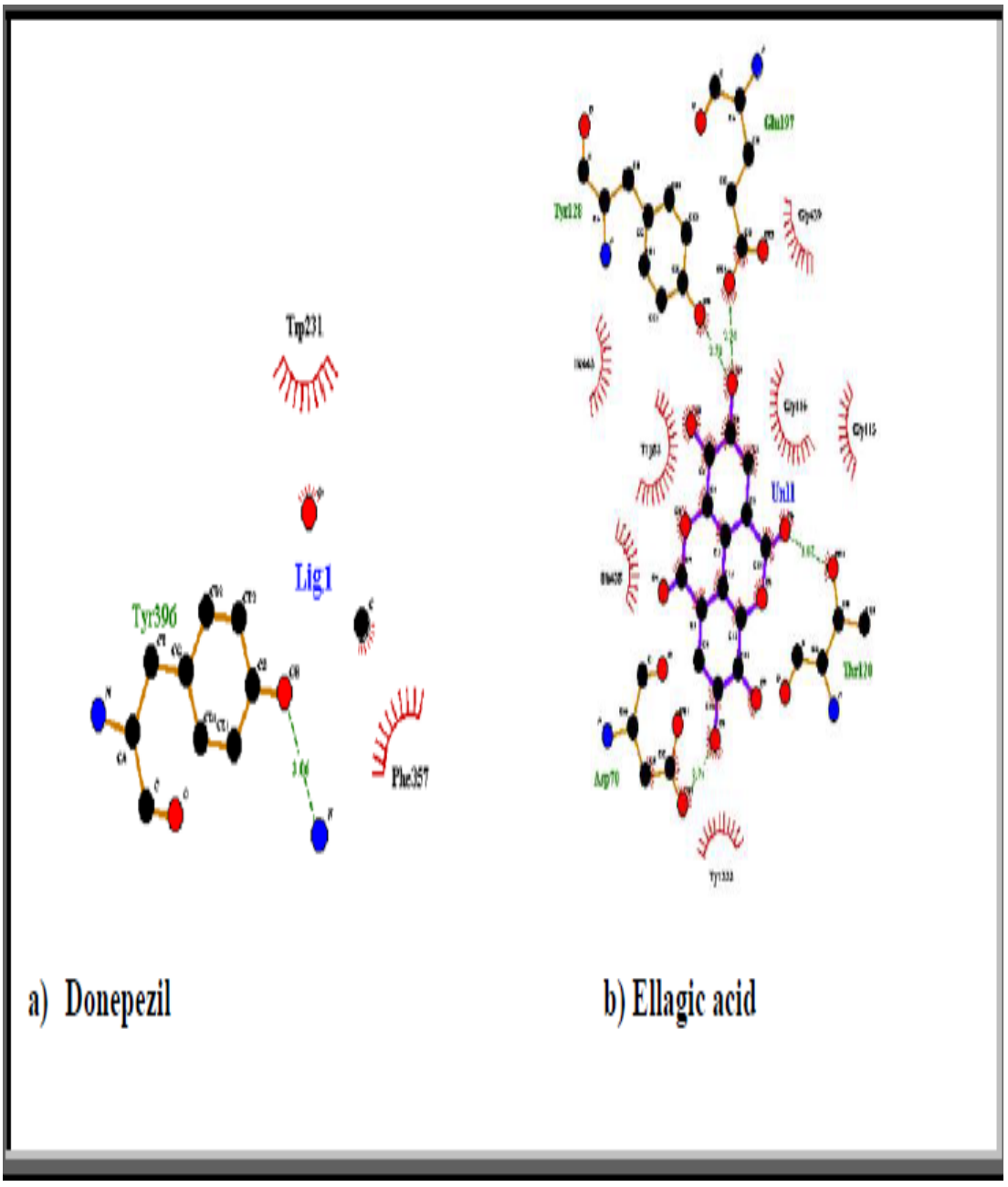


FIGURE 4.16: Interaction of Donepezil and Ellagic acid with receptor protein BChE.

Ellagic acid forms four hydrogen bonds with residues Asp, Thr, Tyr and Glu whereas donepezil forms only one hydrogen bond with Tyr.

Furthermore, ellagic acid shows six hydrophobic interactions with residues Gly439, Gly115, Gly116, Tyr333, His435, Ile443 and Trp53 whereas donepezil shows two interactions with Trp231 and Phe357 as indicated in **Table 4.26**.

TABLE 4.26: Docking analysis Comparison with receptor protein BChE.

S.No	Compound	Binding Energy & No of HBs	Hydrogen Bonding		Hydrophobic Bonding
			Amino Acids	Distance	
1	Donepezil	-9.6 & 1	Tyr396	3.06	Trp231 Phe357
2	Ellagic acid	-10 & 4	Asp70 Thr120 Tyr128 Glu197	2.71 3.02 2.70 2.74	Gly439 Gly115 Gly116 Tyr333 His435 Ile443 Trp53

Chapter 5

Conclusions and Future Prospects

The basic aim of this research was to screen out the active constituents of *Ajuga bracteosa* that could act as potential inhibitors for aceylcholinestrase and butyrylcholinestrase enzymes for the treatment of Alzheimer disease. In silico molecular docking was used for this purpose.

From plant, *Ajuga bracteosa* 21 ligands were selected from literature and databases that showed inhibitory potential. Compounds were docked against receptor proteins AChE and BChE. The structure of ligands was downloaded from PubChem and their energies were minimized using Chem 3D and protein structure was also downloaded from PDB. An online tool, CB Dock was used to dock the compounds. The results were visualized using PyMol and were analyzed using LigPlot+.

Then the compounds were screened out for drug likeliness on the basis of Lipinski rule of 5 and pharmacokinetic properties. After critical analysis of binding scores, Physiochemical properties and ADMET properties, Ellagic acid was selected as a leading compound against both receptor proteins AChE and BChE. Virtual screening results, Physiochemical properties and Pharmacokinetic properties of this compound were compared with FDA approved drug Donepezil. Based on the comparative results it was observed that leading compound showed better binding affinity to respective protein targets with less harmful effects than the standard drug.

5.1 Recommendations

As per the research results, ellagic acid should be explored as a drug candidate for treatment of Alzheimer disease in further *in vitro* and *in vivo* experiments. Previously, *Ajuga bracteosa* was used as an anti-viral, anti-oxidant and anti-inflammatory for this reason its effectiveness should also be tested against Alzheimer disease.

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