In-silico analysis of potential Tau protein kinase inhibitors using docking studies for the treatment of Alzheimer's disease

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Submitted by

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CERTIFICATE

This is to certify that the M. Tech. dissertation entitled "***In-silico* analysis of potential Tau protein kinase inhibitors using docking studies for the treatment of Alzheimer's disease**", submitted by **NIKHIL (2K14/BIO/16)** in the partial fulfilment of the requirements for the award of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by him under my guidance. The information and data enclosed in this dissertation is original and has not been submitted elsewhere for honouring of any other degree.

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DECLARATION

I declare that my major project entitled "***In-silico* analysis of potential Tau protein kinase inhibitors using docking studies for the treatment of Alzheimer's disease**", submitted to Department of Biotechnology, Delhi Technological University as a result of the work carried out by me at "Molecular Neuroscience and Functional Genomic Laboratory", Department of Biotechnology, as major project.

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In silico analysis of potential Tau protein kinase inhibitors using docking studies for the treatment of Alzheimer's disease

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ABSTRACT

Alzheimer's disease is a brain disorder which leads to serious deterioration in many mental functions like memory, orientation, language and judgment. The main characteristic feature of this disease is the formation of senile plaques (mostly β amyloid) and neurofibrillary tangles (formed due to altered tau protein) which results in neuronal destructions. Neurofibrillary tangles are formed due to hyperphosphorylation of tau and its subsequent aggregation. Several kinases are responsible for phosphorylation of tau protein, but in AD brains these kinases are upregulated. Inhibition of multiple kinases may prevent tau aggregation in neurofibrilary tangles and prevent progress of Alzheimer's and its associated neurodegeneration. In this study we targeted GSK3β and CDK5/p25 which play the most prominent role in hyperphosphorylation of tau. Plant derivatives especially flavonoids are suitable candidates for inhibitors as they are nontoxic and possess anti-oxidant properties. Several plant derivatives were selected and autodock4.2 was used to analyse their inhibition potential for GSK3β and CDK5. Flavonoids showing minimum binding energy can be used as potential inhibitors in therapy against Alzheimer's.

CHAPTER 1: INTRODUCTION

Alzheimer's disease (AD) is among most prevalent neurodegenerative disease among elderly people and causes 80% of dementia cases worldwide. It leads to decline in mental, functional, behavioural decline and learning ability (Kumar et al., 2015)

It is a progressive condition initially characterized by short-term impairment of memory, which later evolves in profound physical and cognitive disability. In aging populations of industrialized countries incidence of AD is increasing at a high rate. AD is prevalent in 1% of 60 year olds. Every 5 years it is approximately doubled, reaching to 16% at age of 80 (Silvestrelli et al., 2006).

AD is diagnosed on the basis of clinical features, and confirmed by an examination of brain tissue. It can be categorized in three stages – mild, moderate and severe which involves functional and cognitive decline over a span of 5–8 years. The mild stage lasts 2–3 years and is characterized by symptoms of depression and anxiety along with impairment of short-term memory. In the moderate stage, there is reversal of sleep pattern and patient experiences visual hallucinations. In final and most severe stage there is a notable cognitive decline patient becomes completely dependent on others. There is a linear decline throughout the three stages in the cognitive and functional ability of patients. There is maximum burden on caregiver with the appearance of neuropsychiatric symptoms in patient and decreases a little during the final stage (Gauthier, 2002).

AD is characterized by the presence of cerebral beta-amyloid (Ab) plaques, Neuro fibrilary tangles (NFT) which are made of hyperphosphorylated microtubule-associated protein tau (MAPt) and neuronal loss. NFT are formed partly of a protein called tau, which clumps together to form filaments. The severity of dementia is proportional to the density of these filaments inside neurons in the brain. (Hardy and Selkoe, 2002).Till now there is no definitive cure for AD. The correlation among stages of AD and structures in brain suggest that a therapeutic intervention can stop the progress of disease and pathological changes that occur with it (Silvestrelli et al., 2006).

The cellular and molecular mechanisms which result in the formation of NFTs and beta-amyloid plaques are still not clear and it is still controversial whether these lesions play a peripheral role in the disease or are the primary causative factor. Post-translational modifications in tau have an important role in aggregation of tau linked to AD (Martin et

al.2011). Phosphorylation is the most important modification among these post-translational modifications with 85 putative sites. In AD, excessive phosphorylation of tau results in its aggregation into NFT (Avila, 2006).

The phosphorylation of tau is regulated and depends on a balance between phosphatase and tau kinase. It is suggested that the disruption of this equilibrium is the origin of abnormal phosphorylation of tau and contributes to tau aggregation. Thus, understanding of the modes of regulation of tau phosphorylation is crucial to determine the various causes in formation of tau aggregates and to develop strategies to cope with them in AD. The Inhibition of tau kinases is one of the methods which can prevent tau hyperphosphorylation (Martin et al., 2013). Recent studies reveal that there is increase in activity of glycogen synthase kinase 3β (GSK3β) (also called human tau protein kinase I) in Alzheimer's disease. Similarly, cyclin-dependent kinase 5 (Cdk5)/ P25 also possess higher activity in AD brain (Jagadeesh et al., 2013).

CHAPTER 2: REVIEW OF LITERATURE

In the last century there has been a lot of improvement in the health care which lead to increase in average lifespan of people. It has also increased occurrence of age related disorders such as dementia. Dementia is a chronic and progressive syndrome caused due to different brain illnesses that affect thinking, memory, behaviour and the ability to perform everyday tasks (McKhann et al, 2011). It is overwhelming for people who have it as well as for their families and caretakers. As per WHO it is among the major causes of disability and dependency among old people throughout the world.

2.1 ALZHEIMER'S DISEASE

Dr. Alois Alzheimer a German neuropathologist and psychiatrist described for the first time a dementia like condition which was later called as AD. He described the case of a 51-year-old woman , Auguste D with a 'disease of cerebral cortex,' who had progressive memory loss as well as language impairment, behavioural symptoms, disorientation, (delusions, hallucinations, paranoia), and psychosocial dysfunction. (Alzheimer A., 1907)

Alzheimer's disease is one of the most persistent dementia and contributes to 60-70% of cases. It affects million across the world and leads to social and behavioural handicaps. The estimated cost of treating AD was $604 billion in 2010. These are staggering costs, and alarming since the predicted number of cases which are currently 36 million is set to triple by the year 2050 (Wimo and Prince, 2010). Remarkably, majority of the observations and findings of Dr. Alois Alzheimer play a central role in the understanding we have of AD even after a century.

There are two main hypotheses for Pathological mechanisms of Alzheimer's disease

1. Amyloid hypothesis-the amyloid hypothesis is based on the idea that extracellular amyloid beta (Aβ) deposits (senile plaques) are the fundamental cause of the disease. Gene for amyloid precursor protein is located on chromosome 21; patients with Down syndrome (trisomy 21) almost universally exhibit AD by 40 years of age.
2. Tau hypothesis – Hyperphosphorylated tau begins to pair with other threads of tau. Neurofibrillary tangles are formed inside nerve cell bodies. This leads to disintegration of microtubules, destroying the structure of cells cytoskeleton. This results in malfunction in communication and finally death of cells.

2.2 TAU PROTEIN

Tau protein was discovered in 1975 as it co-purified along with microtubules and was named tau for its ability to induce tubule formation (Weingarten et al., 1975). Later on it was found that tau was a phosphoprotein (Cleveland et al., 1977), further it was shown that tau in a dephosphorylated state shows greater efficiency in promotion of microtubule assembly (Lindwall et al., 1984).

Tau is a mainly but not exclusively neuronal microtubule- associated protein (MAP). Its functions include the stabilization of axonal microtubules (Drubin et al., 1986); role in signal transduction, neurite outgrowth, interaction with the actin cytoskeleton, interactions with the plasma membrane, anchoring of enzymes such as protein kinases and phosphatases, and the regulation of intracellular vesicle transport. Mutations in the tau gene have been linked to several Alzheimer-related dementias, also called as FTDP-17 (front temporal dementia with Parkinsonism related to chromosome 17) (Spillantini et al., 1998). However, the detailed mechanism(s) behind these tau pathologies is yet to be discovered. Since tau is a highly soluble protein, tolerates heat, denaturing agents or acid treatment without losing its biological function, it is counter intuitive that this protein should aggregate into insoluble fibers. In last few years many studies on the structure and assembly of tau, its interaction with microtubules, phosphorylation by various kinases, and have been carried out. Tau gets disassociated from its natural partner the microtubule after phosphorylation. It could be an important step in generation of protein for the assembly of paired helical filaments (PHFs) due to increase the soluble pool of tau. However, the assembly itself appears to depend mainly on other factors like conformation, oxidation, and nucleation by other components (Friedhoff et al., 2000).

In comparison to amyloid plaques the distribution of neurofibrillary deposits is more correlated with the clinical progression of the disease. In this regard, tau deposits have a similar diagnostic value to the loss of synapses. In addition the amount of tau in the cerebrospinal fluid increase in Alzheimer’s which creates a potential route to early diagnosis.

The regulation of functions of tau is related to its phosphorylation state. The longest isoform of human brain tau consist of five Tyrosine residues and 80 Serine or Threonine residues, and thus nearly 20% of the tau protein can undergo phosphorylation. Dozens of protein kinases have been shown to phosphorylate tau in

vitro, still in a physiologically relevant context the number of kinases that can regulate the phosphorylation state of tau is small (Stoothoff et al., 2005).

2.3 TAU PROTEIN KINASES

Kinases are part of class of enzyme called “transferases” because they can transfer phosphate group from donor molecules, like GTP or ATP, to different substrates. The catalytic core is the most conserved in all kinases and phosphorylation occurs in this region. This active domain has a GTP/ATP binding pocket and a site where the substrate which is to be phosphorylated is kept. Phosphate transfer occurs, when the substrate and energy donor come in contact with the kinase. Activity of kinases is regulated by post translational modifications like phosphorylation, by modification of specificity of the kinase, and by intracellular distribution of the kinase. Tau protein kinases are grouped into three types: tyrosine protein kinases (TPK), protein kinases non-PDPK, proline-directed protein kinases (PDPK).

2.3.1 GSK3β

GSK3β structure consists of two isoforms such as α and β which are coded by different genes; still they display about 85% homology in sequence (Shaw et al., 1998; Woodgett, 1990). Alternative splicing of GSK3β isoform of exon 8A results in two isoforms 1 and 2 which differ due to insert of 13 amino acid between leucine 303 and valine 304 in the 2^{nd} isoform. 2^{nd} isoform is responsible for only 15% of overall GSK3 activity and displays lesser activity of tau phosphorylation than the 1^{st} isoform. 1^{st} isoform is found in axons as well as neuronal soma but 2^{nd} isoform is present in neuronal soma alone (Mukai et al., 2002).

GSK3 was initially discovered for to its role in the metabolism of glycogen (Welsh & Proud, 1993). GSK3β is also known for its function in cell proliferation, embryonic development, neural functions, apoptosis, oncogenesis and immune pathways (Beurel et al., 2010; Hooper et al., 2008). GSK3 activity is inhibited by Phosphorylation of serine 21(for GSK3α isoform), serine residues 9 and 389 (for GSK3β isoform) (Stambolic & Woodgett, 1994). GSK3 activity increases by phosphorylation at tyrosine 279 (for GSK3α isoform) (Wang et al., 1994) and at tyrosine 216 (for GSK3β isoform). (Ali et al., 2001; Cole et al., 2004).

Efficient phosphorylation by GSK3β requires a substrate to have prior phosphorylation at a site which is 4 amino acids upstream of the phosphorylation target site of GSK3β, under a process termed as “substrate priming” (Dajani et al., 2001).

2.3.1.1. ROLE OF GSK3β IN AD

Systems of Mammalian cell culture have been very useful in elucidation of the signaling pathways which regulate tau phosphorylation and the consequences of specific phosphorylation events on function of tau. GSK3β is one of the protein kinase which has gained significant attention as a tau kinase. GSK3β phosphorylates tau protein at 42 sites and 29 out of them are also found phosphorylated in AD brains (Hanger et al., 2009; Sergeant et al., 2008). Initial co-transfection studies of tau and GSK3β into nonneuronal cells resulted in a great increase in phosphorylation of tau at many epitopes. Co-expression of GSK3β along with tau impaired the binding ability of tau to microtubules (Johnson et al., 1999).

GSK3β might also have a role in regulation of degradation of tau protein. Degradation of tau is most likely done by the proteasome (David et al., 2002), and aggregation of tau in oligodendroglial cells is a result of proteasomal inhibition along with a rise in phosphorylation of tau (Goldbaum et al., 2003).

2.3.1.2. INHIBITORS OF GSK3β

Lithium is the first characterized inhibitor of GSK3β (Klein & Melton, 1996). Many Bipolar disorders are treated with lithium. It is weak inhibitor of GSK3β. Lithium being a monovalent cation, can easily pass blood brain barrier (BBB). Lithium competes with magnesium for the ATP binding pocket and directly inhibits GSK3β (Ryves & Harwood, 2001). The common most GSK3β inhibitors are: Indirubins (Leclerc et al., 2001), paullones (Leost et al., 2000), thiadiazolidinones (Martinez et al., 2002), anilinomaleimides (Smith et al., 2001). Many of these compounds also inhibit CDK5 and CDK2 and do not differentiate between GSK3β and CDK5 (Bhat et al., 2004).

2.3.2. CDK5

CDK5 works along with its co-activator named p29/p25 andp39/p35. Cdk5 belongs to cyclin-dependent kinase family, though it is not involved in cell cycle regulation. Inbrain development and function Cdk5/p35 has a dominant role (Smith et al., 2002). This kinase shows highest activity in neurons which is due to the expression of its

regulator p35 selectively in these cells. The monomeric form of CDK5 is inactive without its interaction with co-activator. P35 shares nearly 57% of homology with p39 which is expressed fetally and its expression decreases gradually after p35 is expressed (Dhariwala and Rajadhyaksha, 2008). CDK5 activity is found to be restricted to neurons (Guidato et al., 1998; Paglini and Caceres, 2001), because of the distribution of its co-activators p39 and p35 in nervous system (Humbert et al., 2000).

CDK5 is very important for the development of the central nervous system (CNS) and is plays the role in regulating the neuronal cytoskeleton dynamics (Smith, 2003; Hallows et al., 2003), vesicular transport (Barclay et al., 2004), neuritic outgrowth (Nikolic et al., 1996), and synaptic functions (Samuels et al., 2007).

2.3.2.1. ROLE OF CDK5 IN AD

When complexed with p25, Cdk5 is able to phosphorylate tau at similar epitopes which are phosphorylated in mitosis. Cdk5/p25 might be able to perform (mitotic-like) phosphorylation of tau in Alzheimer's disease brain (Hamdane et al., 2003). The p25/p35 ratio and level of the CDK5/p25 hyperactive complex are increased in AD brains, (Camins et al., 2006). CDK5 plays a role in promotion neuronal apoptosis (Cheung et al., 2008; Li et al., 2002).Aβ peptide-resultant toxicity is reduced by CDK5 inhibition(Lopes et al., 2010; Changet al., 2010) whereas over expression of p35 results in Aβ-induced apoptosis (Utreras et al., 2009). CDK5 can be a promising therapeutic target for Alzheimer's diseaseCDK5 Silencing in transgenic mice leads to reduction in formation of NFT (Piedrahita et al., 2010).

2.3.2.2. INHIBITORS OF CDK5

CDK5 inhibitors: Two related compounds: (R)-roscovitine and (R)-CR8 show specific inhibition CDK5 (Bettayeb et al., 2008).(R)-roscovitine is important in AD since it is able to cross BBB (Meijer and Raymond, 2003) and already used in clinical trials for breast cancer and, lymphoma and non-small cell lung cancer (Ribas et al., 2006).

2.4. FLAVONOIDS

Flavonoids are a vast group of polyphenolic compounds ubiquitously found in plants which have a benzo-γ-pyrone structure. Flavonoids are formed by phenyl propanoid pathway. Pharmacological activities have been seen in secondary metabolites which are

of phenolic nature including flavonoids (Mahomoodally et al. 2005, Pandey , et al. 2007). Plants synthesize flavonoids to defend themselves from microbial infection (Dixon et al. 1983).Their activities depend on their structure. The chemical properties of flavonoids depend on their degree of hydroxylation, structural class, degree of polymerization; conjugations and other substitutions (Kelly et al. 2002). They have been used traditionally for their health benefits and antioxidant properties. Flavonoids have functional hydroxyl groups which help in chelating metal ions and scavenging of free radicals (Kumar et al., 2013).

The main types of flavonoids include (1) flavonols found in broccoli, onions, leeks (e.g., quercetin, kaempferol); (2) flavones found in celery and parsley(e.g., luteolin, apigenin); (3) isoflavones mainly present in soy and soy products (e.g., genistein, daidzein); (4) flavanones mainly present in citrus fruits (e.g., naringenin, hesperetin); (5) flavanols which are found in red wine, green tea and chocolate (e.g., epicatechin, catechin, epigallocatechin, epigallocatechin-3-gallate (EGCG); and finally (6) anthocyanidins which are found in berry fruits and also red wine (e.g., malvidin, cyanidin, pelargonidin) (Pietta2000).

2.4.1. Luteolin

The chemical name of Luteolin is 3′, 4′, 5, 7-tetrahydroxyflavone, it is a flavonone, a type flavonoid that is commonly found in different plants including vegetables, fruits, and herbs (Lin et al., 2008). Traditional Chinese medicines have been using luteolin for treatment of diseases such as inflammatory disorders, hypertension, and cancer. Its appearance is yellow crystalline. Dietary sources include broccoli, celery, parsley, green pepper, dandelion, thyme, chamomile tea, perilla, carrots, olive oil, rosemary, peppermint, oregano, navel oranges. Its chemical formula is $C_{15}H_{10}O_6$ and molar mass is 286.24 g/mol

2.4.2. Morin

The chemical name of Morin is 2-(2, 4-dihydroxyphenyl)-3, 5, 7-trihydroxychromen-4-one is a yellow chemical compound. It can be isolated from *Maclura tinctoria* (old fustic), *Maclura pomifera* (Osage orange), and from leaves of *Psidium guajava* (common guava). Its Chemical formula is $C_{15}H_{10}O_7$ and molar mass is 302.2357 g/mol.

2.4.3. Myricetin

The chemical name of Myricetin is 3, 5, 7-Trihydroxy-2-(3, 4, 5-trihydroxyphenyl)-4-chromenone is a member of the flavonoid class of compounds, with antioxidant properties. It is commonly extracted from fruits, vegetables, nuts, tea, berries and also found in red wine. Its Chemical formula is $C_{15}H_{10}O_8$ and molar mass is 318.24 g/mol.

2.4.4. Quercetin

Quercetin is 2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxy-4H-chromen-4-one is a major flavonoid present in the human food. Quercetin has anti-allergic and anti-inflammatory properties. It has chemical formula $C_{15}H_{10}O_7$ and molar mass is 302.236 g/mol.

2.5. Kenpaullone

The chemical name of kenpaullone is 9-Bromo-7, 12-dihydroindolo [3, 2-d] [1] benzazepin-6(5H)-one. Its molecular weight is 327.18 g/mol and chemical formula is $C_{16}H_{11}BrN_2O$. Kenpaullone is an ATP-competitive inhibitor. It inhibits GSK-3β (Glycogen synthase kinase 3β) as well as several CDKs (Cyclin-dependent kinases). Kenpaullone is used in study of cell cycle regulators. Kenpaullone inhibits of p35, ERK 2, cyclin E etc.

2.6. Molecular Docking

An important tool for understanding the binding interactions between a ligand and its target protein receptor is Computer-aided docking. It has proven to be cost effective, time saving and reliable method in search of lead compounds (Schneider and Böhm, 2002). In the past few years it has been become an important part of drug discovery. Computational analysis tools like Auto-Dock provide the benefit of delivering novel drug candidates at quicker pace and low cost (Warren et al., 2006). Autodock uses stochastic Lamarckian genetic algorithm for computation of ligand conformations and simultaneously minimizes its scoring function (Morris et al., 2009).

CHAPTER 3:

MATERIALS AND METHODOLOGY

3.1. Bioinformatics software and tools:

Bioinformatics can enable efficient drug discovery by decreasing the cost and time involved in search of the optimum drug. Through combined use of online databases and standalone software we can find a ligand against our target protein easily. Further docking analysis can show the potential of ligand to interact with Protein. This can pave a way to development of a novel drug.

3.1.1. Online servers used:

- http://www.rcsb.org/
- http://zinc.docking.org/
- http://sts.bioe.uic.edu/castp/
- http://uniprot.org
- https://www.ncbi.nlm.nih.gov/
- http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp

3.1.2. Software's used:

- Swiss-Pdb Viewer 4.10
- Auto Dock 4.2
- Auto DockVina 1.1.2
- Chimera 1.11
- MGL Tools 1.5.6
- Python 2.7.6

3.2. METHODOLOGY

3.2.1. Retrieval of protein structure

The crystal structure of Cyclin dependent kinase (PDB ID 1H4L) and Glycogen synthase kinase 3β (PDB ID 1H8F) was retrieved from Protein Data Bank (*http://www.rcsb.org/)*in PDB text format.

The Zinc database site (*http://zinc.docking.org/)* was used to extract the structures of four plant derivatives along with Kenpaullone in mol2 format.

3.2.2. Active Site Prediction:

CASTp server was used for prediction of active sites in crystal structure of GSK3β and CDK5. It predicts active sites of proteins associated with structural pockets and cavities. It can identify and measure surface as well as interior cavities. The active sites residues present in GSK3β and CDK5 were identified after selecting the structural cavity.

3.2.3. Phylogenetic Analysis:

MEGA6 software is tool developed for genetic and evolutionary analyses of protein and DNA sequences. It can help in analysing the evolutionary relationship of genes and proteins among different species. Multiple sequence alignment of tau protein of six species was done to see which regions are conserved. Alignment of the 9 isoforms of human tau protein was also carried out.

3.2.4. Prediction of phosphorylation sites:

NetPhosK 1.0 Server is used to produce neural network predictions of eukaryotic protein phosphorylation sites which are kinase specific. Presently the NetPhosK can cover the following kinases: GSK3, Cdk5, p38 MAPK,Cdc2, CaM-II, PKA, PKC,CKII, ATM, PKG,CKI, PKB, DNA PK, INSR, EGFR, RSK, and Src.The FASTA sequence of human tau protein was used to find out the phosphorylation sites in the human tau protein.

3.2.5. Preparation of protein molecules:

Standalone software Chimera was used to prepare protein molecules for docking. The dock prep tool in Chimera helps in optimizing the structure of protein by removal of water molecules, addition of hydrogen atoms and neutralizes the charges. It can remove any other structure bound to molecule. It can convert in pdb format as well as mol2 format.

3.2.6. Docking Studies:

Auto Dock 4.2 is a Docking tool used to study the interaction between protein target and ligand. It employs the empirical free energy scoring function and Lamarckian Genetic Algorithm. The prepared and optimized structures of target protein and ligand are converted into pdbqt format and docking is performed through command line. LIGPLOT1.4.5 was use to depict the protein-ligand interactions in form of diagrams.

3.2.7. Lipinski Filter

Lipinski rule of 5 can be used in distinguish drug like molecules. The structure of ligand molecules was downloaded from Zinc database in mol2 format and submitted to server *(http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp*). It is used to predict probability of success or failure due to drug likeness for molecules complying with two or more of the following rules:

- Molecular mass should be lower than 500 Dalton
- It should have High lipophilicity (LogP less than 5)
- Hydrogen bond donors should be Less than 5
- Hydrogen bond acceptors should be Less than 10
- Its Molar refractivity must be 40-130

CHAPTER 4: RESULTS AND DISCUSSION

4.1. PROTEIN STRUCTURES AND SEQUENCES

Crystal structure of Glycogen synthase kinase 3β with PDB ID-1H8F and Cyclin dependent kinase CDK5 with PDB ID-1H4L was downloaded. We searched 9 isoforms of human Tau protein and Tau protein sequence of six species using BLAST tool. Tau protein sequences were downloaded in FASTA format.

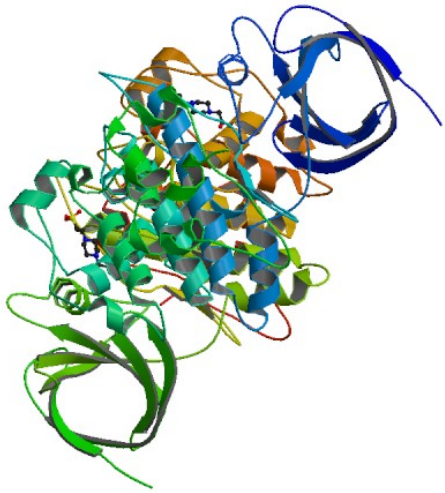


Figure 1: 3D structure of target molecule (PDB ID 1H8F)

(Glycogen synthase kinase 3β)

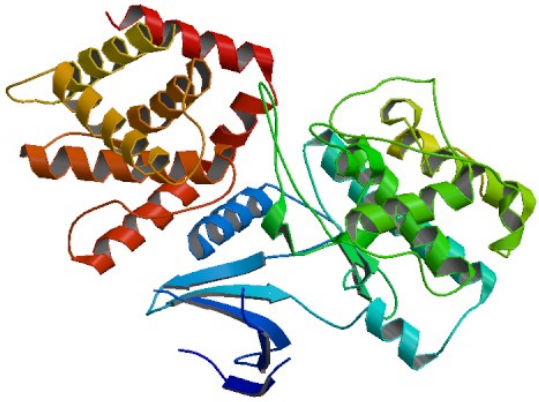


Figure 2: Structure of target molecule (PDB ID 1H4L)

(Cyclin dependent kinase CDK5)

4.2. ACTIVE SITES

4.2.1. Active sites in GSK3β

The CASTp server gives the active site region in GSK3β protein. The PDB ID 1H8F was entered in the server. Active sites can be target for potential drugs. The active site present in the protein is shown in blue colour. It contains the following amino acids- TYR^{140}, ALA^{143}, ARG^{144}, TYR^{146}, SER^{147}, ARG^{148}, LYS^{150}, GLN^{151}, THR^{152}, LEU^{153}, PRO^{184}, GLN^{185}, SER^{215}, TYR^{216}, ARG^{220}, TYR^{221}, ARG^{223}, LEU^{227}, ILE^{228}, GLY^{249}, LEU^{250}, LEU^{252}, GLY^{253}, GLN^{254}, PRO^{255}, PRO^{258}, GLY^{259}, ASP^{260}, SER^{261}, GLY^{262}, GLN265 and ARG^{306}.

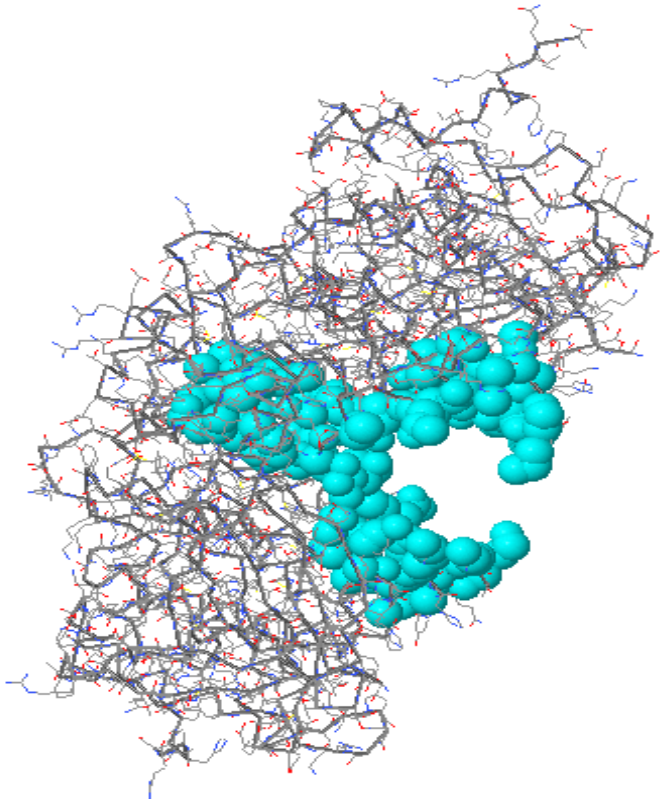


Figure 3: Active Site cavity in GSK3β predicted by CASTp server

4.2.2. Active sites in CDK5

The CASTp server gives the active site region in CDK5 protein. The PDB ID 1H4L was entered in the server. The active site present in the protein is shown in green colour. It contains the following amino acids-GLN[2], TYR[4], GLU[5], LYS[6], LEU[7], GLU[8], LYS[9], ILE[10], TYR[15], GLY[16], THR[17], VAL[18], PHE[19], LYS[20], LYS[22], ARG[24], HIS[27], ILE[29], ALA[31], LEU[32],LYS[33], ARG[34], VAL[35], ARG[36], LEU[37], ASP[38], GLY[43], VAL[44], PRO[45], SER[47], ARG[50], GLU[51], VAL[64], LEU[70], SER[72], ASP[73], LYS[75], THR[77], PHE[80],GLU[81], PHE[82], CYS[83], ASP[84], GLN[85], ASP[86], LYS[88], TYR[90], PHE[91], ASP[92], SER[93], ASP[126], LYS[128], GLN[130], ASN[131], LEU[133], ILE[134], ASN[135],ARG[136], ALA[143], ASP[144], GLY[146], LEU[147], CYS[157], TYR[158], SER[159], ALA[160], GLU[161], VAL[162], THR[164], LEU[165], TRP[166], TYR[167], ARG[168],VAL[172], LEU[173], PHE[174], GLY[175], LYS[177], GLN[188], GLY[189], TRP[190], GLN[193], ALA[199], ARG[200], PRO[201], GLY[205], ASN[206], ASP[207], VAL[208],ASP[209], LEU[212], TYR[231], ASP[235], LYS[237], ASN[239], GLU[240], ILE[241], SER[242], TYR[243], PRO[244], LEU[245], LYS[246], LEU[249], LYS254 and TRP[258].

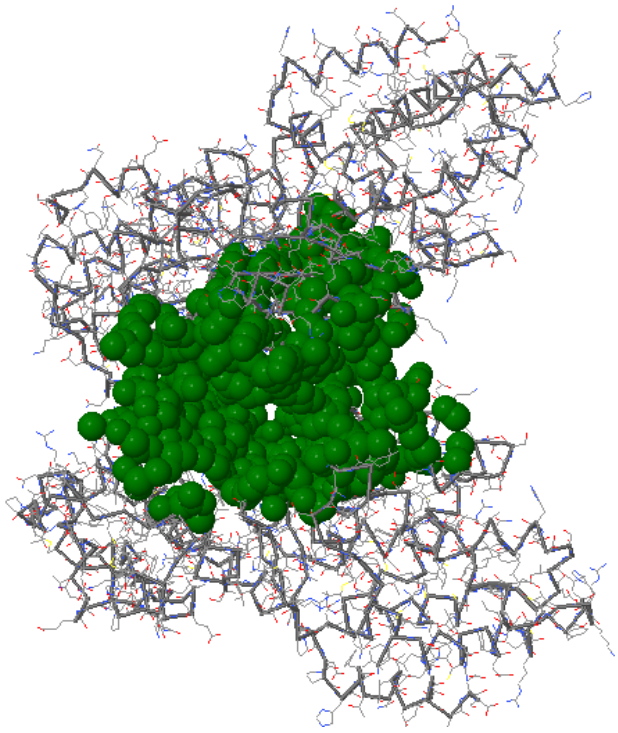


Figure 4: Active Site cavity in CDK5 Predicted by CASTp server

4.3. MULTIPLE SEQUENCE ALIGNMENT

Multiple sequence alignment of tau protein sequences among six species- *Homo sapiens* (Human), *Mus musculus* (Mouse), *Rattus norvegicus* (Rat), *Bos tauru* (Bovine), *Capra hircus* (Goat) and *Macaca mulatta* (Rhesus macaque) and among nine isoforms of human tau was done using MEGA6 to see the conserved sequences present in tau protein. A phylogenetic tree using UPGMA method was constructed. It showed that Human tau protein is evolutionarily closer to tau protein found in Rhesus macaque as compared to other species.

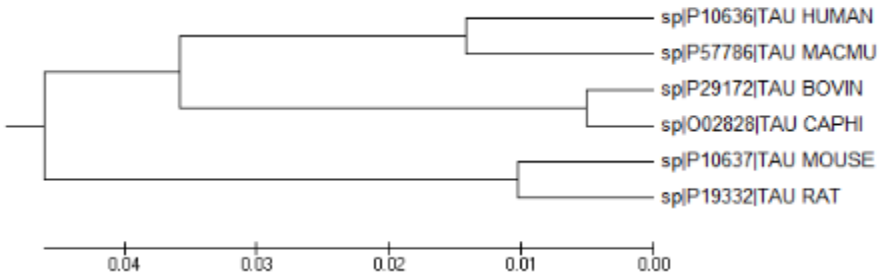


Figure 5a: Phylogenetic tree of tau protein among six species.

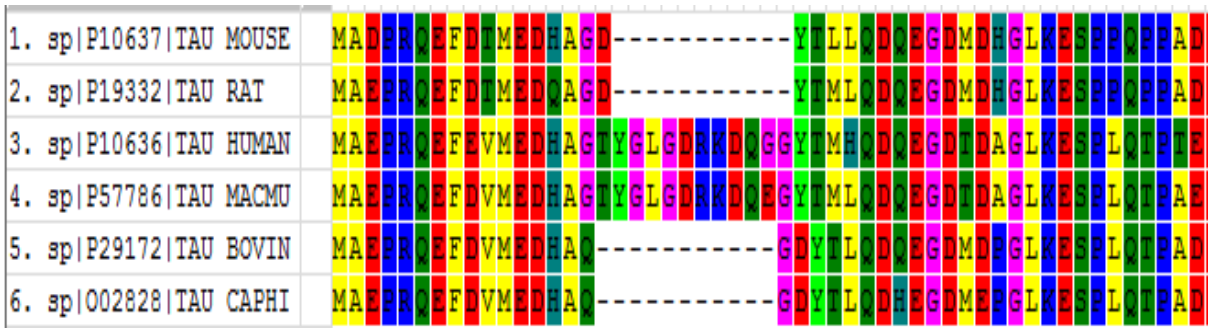


Figure 5b: MSA of tau protein of six species.

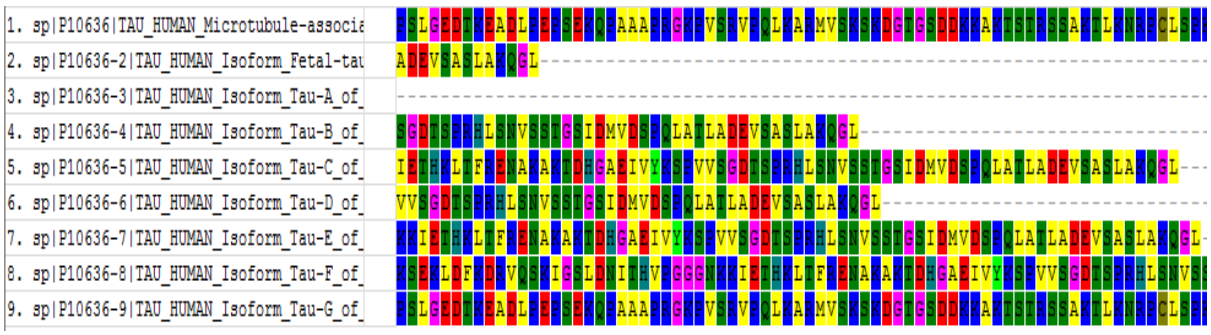


Figure 5c: MSA of 9 isoforms of human tau protein.

4.3. PHOSPHORYLATION SITES IN TAU PROTEIN

Prediction of phosphorylation sites in human tau protein was done using NetPhosK server to see which sites are prone to phosphorylation. Human tau protein contains 57 serine sites, 21 tyrosine sites and 4 tyrosine sites. It is predicted that serine dependent kinases will play a greater role in the phosphorylation of tau protein.

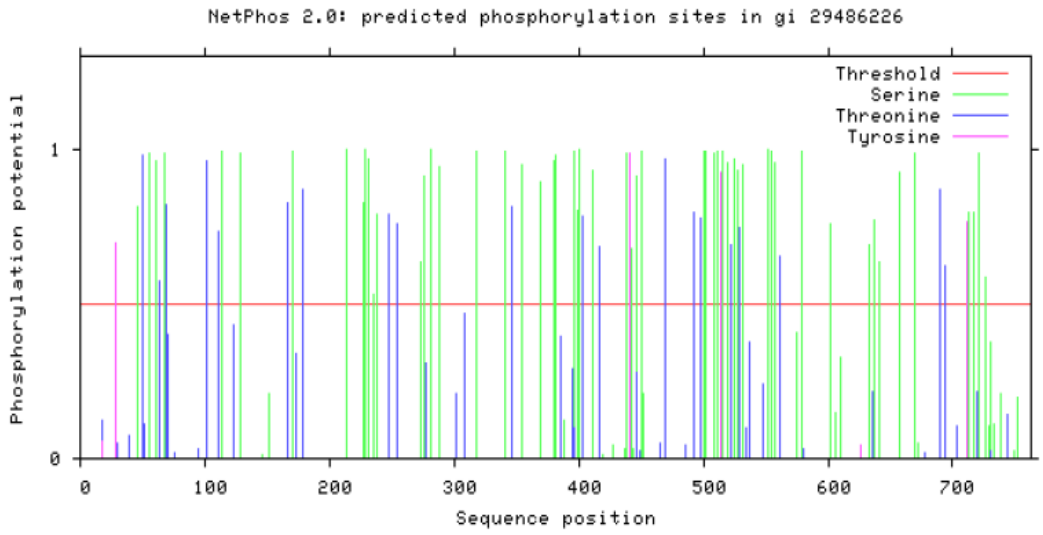


Figure 6a: Prediction of phosphorylation sites in human tau protein

```
  758 gi_29486226
MAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKESPLQTPTEDGSEEPGSETSDAKSTPTAEDVTAPLV      80
DEGAPGKQAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQEPESGKVVQEGFLREPGPPGLSHQLMSGMPGAPLLP     160
EGPREATRQPSGTGPEDTEGGRHAPELLKHQLLGDLHQEGPPLKGAGGKERPGSKEEVDEDRDVDESSPQDSPPSKASPA     240
QDGRPPQTAAREATSIPGFPAEGAIPLPVDFLSKVSTEIPASEPDGPSVGRAKGQDAPLEFTFHVEITPNVQKEQAHSEE     320
HLGRAAFPGAPGEGPEARGPSLGEDTKEADLPEPSEKQPAAAPRGKPVSRVPQLKARMVSKSKDGTGSDDKKAKTSTRSS     400
AKTLKNRPCLSPKHPTPGSSDPLIQPSSPAVCPEPPSSPKYVSSVTSRTGSSGAKEMKLKGADGKTKIATPRGAAPPGQK     480
GQANATRIPAKTPPAPKTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRL     560
QTAPVPMPDLKNVKSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSVQIVYKPVDLSKVTSKCG     640
SLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDT     720
SPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAKQGL                                               800
.........................Y.............S...T.....S....S.T....ST...........              80
...................TT........T.S.............S.............................              160
......T...S......T.................................S............SS...S..S..S..          240
.......T.....TS.................S..S.....S.....S.............................S..          320
...................S....T........S.............S..........S.S.............S..SS          400
..T.......S....T.....................S..Y.S...S...S..................T..........          480
...........T.....T..SS.....S...S.YSS..S..T..S.S.T.S.....................S.SS..S..          560
.T................S.....................S.............................S...S...          640
S................S..........S...................T...T................Y.S...S...          720
S....S.............................                                                   800

Phosphorylation sites predicted:        Ser: 57 Thr: 21 Tyr: 4
```

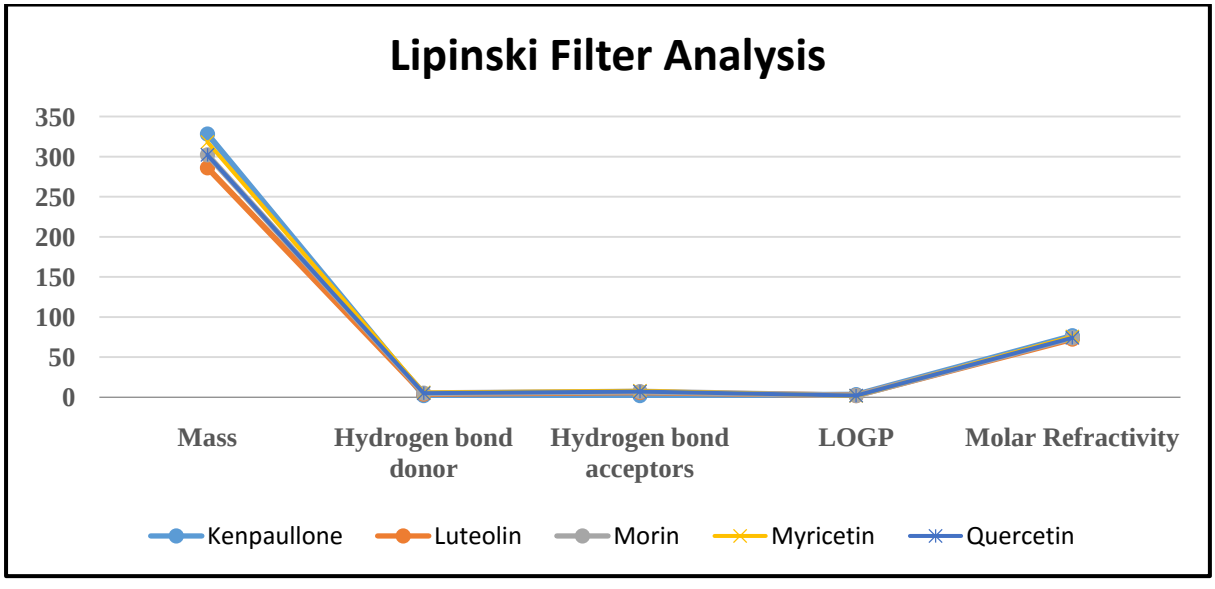
Figure 6b: Phosphorylation sites in human tau protein

4.4. PROPERTIES OF LIGANDS

Properties of selected ligands were analysed based on Lipinski filter. Properties like Mass, lipophilicity, hydrogen bond acceptors, hydrogen bond donors and molar refractivity are used to predict the drug like characteristics of molecules. All the selected Flavonoids meet the criteria for being a drug according to Lipinski's rule of five.

Properties	Kenpaullone	Luteolin	Morin	Myricetin	Quercetin
Mass	328.000	286.000	302.000	318.000	302.000
Hydrogen bond donor	2	4	5	6	5
Hydrogen bond acceptors	2	6	7	8	7
LOGP	3.175060	2.12500	2.01090	1.716500	2.010900
Molar Refractivity	76.508797	72.478676	74.05076	75.715279	74.050476

Table 1: Lipinski Filter analysis



Graph 1: Analysis of Flavonoids on the basis of Lipinski Rule

4.5. DOCKING STUDIES

Autodock 4.2 was used to study the interaction between GSK3β, CDK5 and Flavonoids. Its search function was based on Lamarckian Genetic Algorithm (LGA). Two and three dimensional interactions between ligand and protein were obtained by LIGPLOT+. Further, Table 2 highlights the docking studies calculation score.

Ligands	Target Protein	Est. Free Energy of Binding	Est. Inhibition Constant	Est. Intermolecular Energy	vdW+Hbond+desolv Energy	Electrostatic Energy	Est. Internal Energy	Torsional Free Energy
Kenpaullone	CDK5 (PDB ID 1H4L)	**-3.25 kcal/mol**	**4.13 mM**	**-3.25 kcal/mol**	**-2.90 kcal/mol**	**-0.35 kcal/mol**	**+0.00 kcal/mol**	**+0.00 kcal/mol**
	GSK3beta (PDB ID 1H8F)	**-2.32 kcal/mol**	**20.04 mM**	**-2.32 kcal/mol**	**-2.09 kcal/mol**	**-0.23 kcal/mol**	**+0.00 kcal/mol**	**+0.00 kcal/mol**
Luteolin	CDK5 (PDB ID 1H4L)	**-3.03 kcal/mol**	**6.06 mM**	**-4.52 kcal/mol**	**-3.30 kcal/mol**	**-1.22 kcal/mol**	**-0.95 kcal/mol**	**+1.49 kcal/mol**
	GSK3beta (PDB ID 1H8F)	**-3.83 kcal/mol**	**1.56 mM**	**-5.32 kcal/mol**	**-4.00 kcal/mol**	**-1.32 kcal/mol**	**-0.92 kcal/mol**	**+1.49 kcal/mol**
Morin	CDK5 (PDB ID 1H4L)	**-2.45 kcal/mol**	**16.07 mM**	**-4.24 kcal/mol**	**-3.32 kcal/mol**	**-0.92 kcal/mol**	**-2.38 kcal/mol**	**+1.79 kcal/mol**
	GSK3beta (PDB ID 1H8F)	**-1.89 kcal/mol**	**40.93 mM**	**-3.68 kcal/mol**	**-2.55 kcal/mol**	**-1.14 kcal/mol**	**-1.56 kcal/mol**	**+1.79 kcal/mol**
Myricetin	CDK5 (PDB ID 1H4L)	-2.36 kcal/mol	18.54 mM	-4.45 kcal/mol	-3.71 kcal/mol	-0.74 kcal/mol	-2.80 kcal/mol	+2.09 kcal/mol
	GSK3beta (PDB ID 1H8F)	-2.15 kcal/mol	26.37 mM	-4.24 kcal/mol	-3.40 kcal/mol	-0.84 kcal/mol	-3.37 kcal/mol	+2.09 kcal/mol
Quercetin	CDK5 (PDB ID 1H4L)	-1.30 kcal/mol	110.78 mM	-3.09 kcal/mol	-2.51 kcal/mol	-0.58 kcal/mol	-2.61 kcal/mol	+1.79 kcal/mol
	GSK3beta (PDB ID 1H8F)	-2.33 kcal/mol	19.74 mM	-4.12 kcal/mol	-3.23 kcal/mol	-0.89 kcal/mol	-2.60 kcal/mol	+1.79 kcal/mol

Table 2: Autodock calculations

GSK3β interaction with Kenpaullone

The interaction of GSK3β with Kenpaullone is shown in Figure 7a. 3D and 2D interaction using LIGPLOT+ is shown in Figure 7b. The minimum estimated free energy of binding calculated using autodock was -2.32 kcal/mol. The most common residues interacting between Kenpaullone and GSK3β are SER^{261}, ARG^{223}, GLY^{262}, GLY^{259}, ARG^{220}, TYR^{216}, ARG^{223}, ASP^{260} and CYS^{218}.

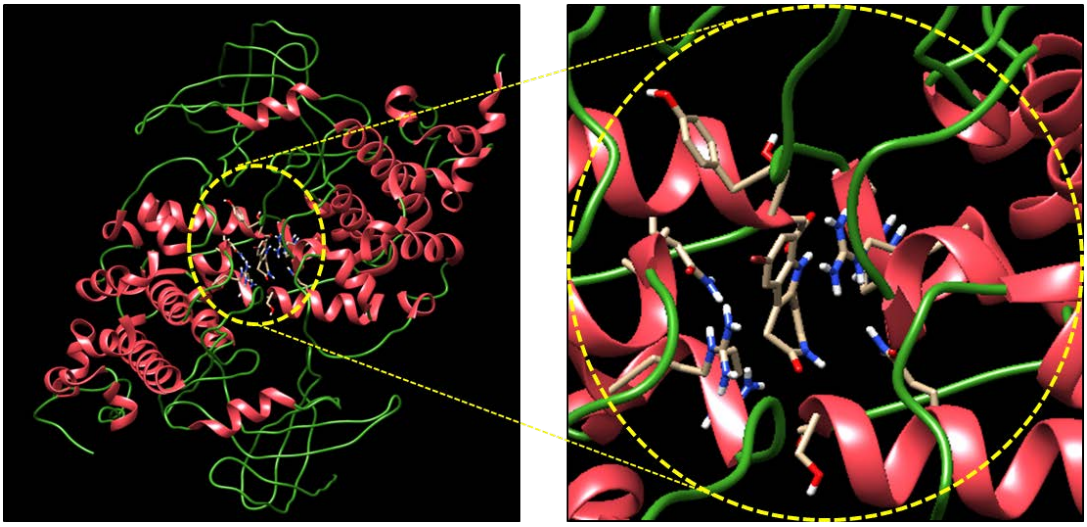


Figure 7a: GSK3β interaction with Kenpaullone

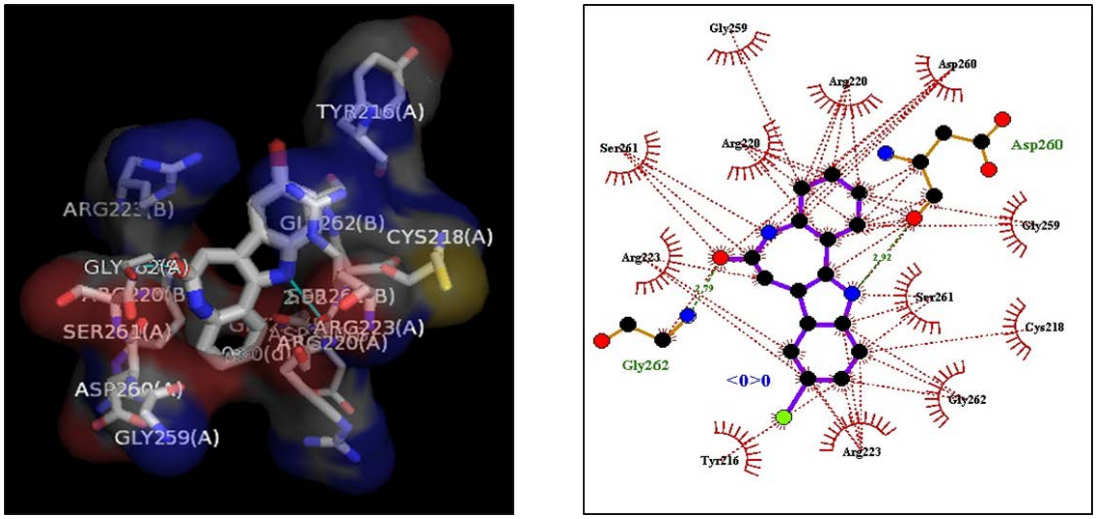


Figure 7b: 3D and 2D interaction between Kenpaullone and GSK3β.

GSK3β interaction with Luteolin

The interaction of GSK3β with Luteolin is shown in Figure 8a. 3D and 2D interaction using LIGPLOT+ is shown in Figure 8b. The minimum estimated free energy of binding calculated using autodock was -3.83 kcal/mol. The most common residues interacting between Luteolin and GSK3β are ARG^{223}, TYR^{216}, SER^{261}, GLN^{265}, ASP^{260}, TYR^{222}, GLY^{259}, ARG^{220}, CYS^{218}, ALE^{217} and GLY^{262}.

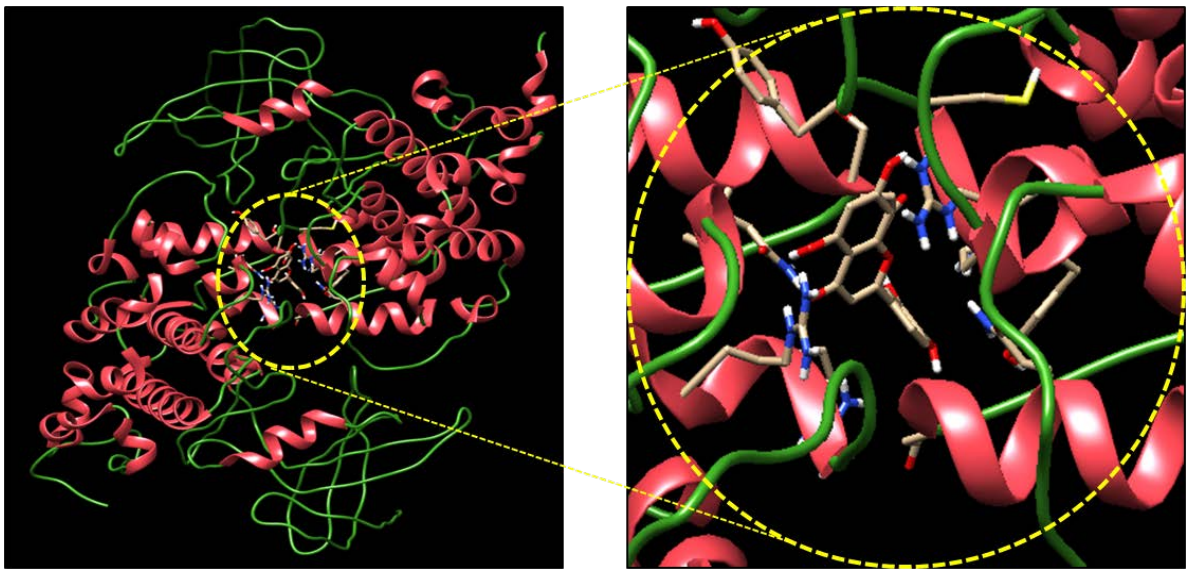


Figure 8a:-GSK3β interaction with Luteolin

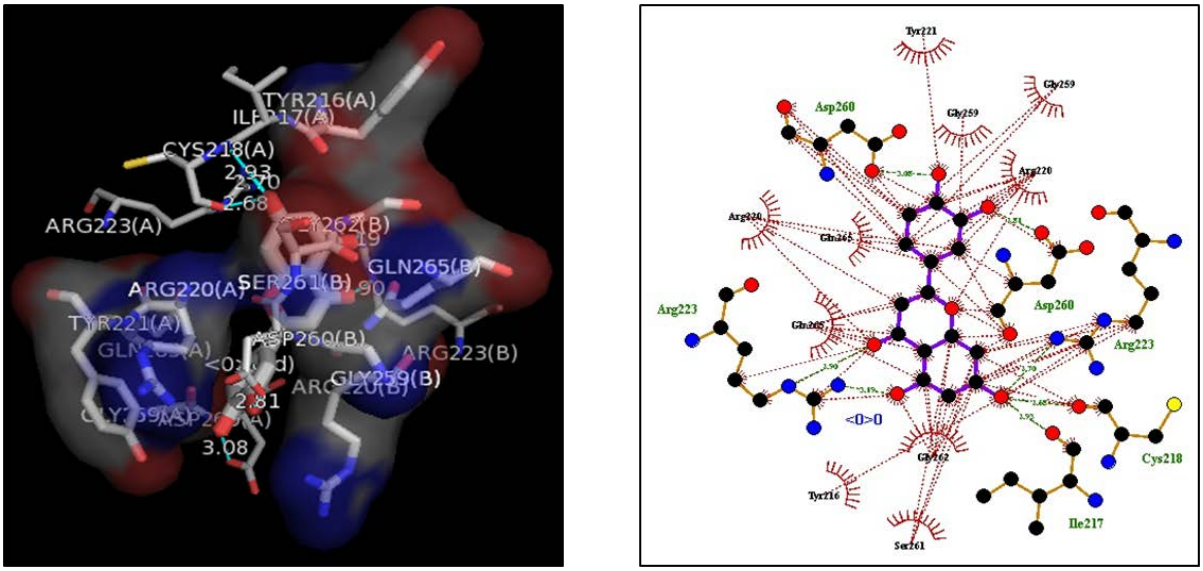


Figure 8b: 3D and 2D interaction between Luteolin and GSK3β.

GSK3β interaction with Morin

The interaction of GSK3β with Morin is shown in Figure 9a. 3D and 2D interaction using LIGPLOT+ is shown Figure 9b. The minimum estimated free energy of binding calculated using autodock was -1.89 kcal/mol. The most common residues interacting between Morin and GSK3β are GLN^{265}, GLY^{259}, ILE^{217}, CYS^{218}, ARG^{223}, SER^{261}, TYR^{216}, GLY^{262}, ASP^{260}, TYR^{221} and ASP^{220}.

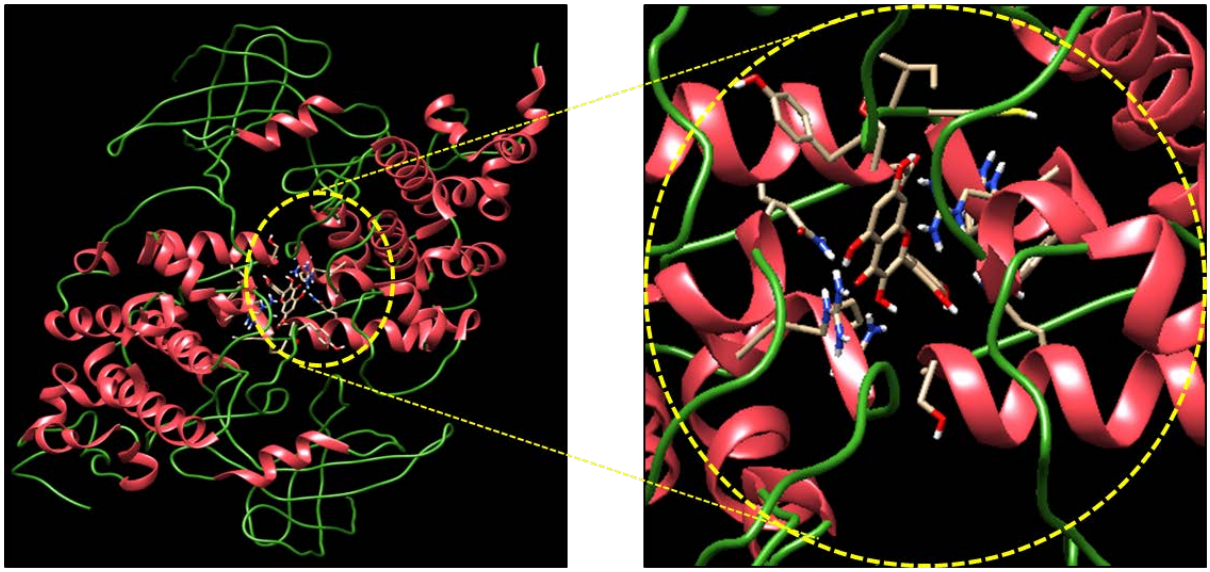


Figure 9a: GSK3β interaction with Morin

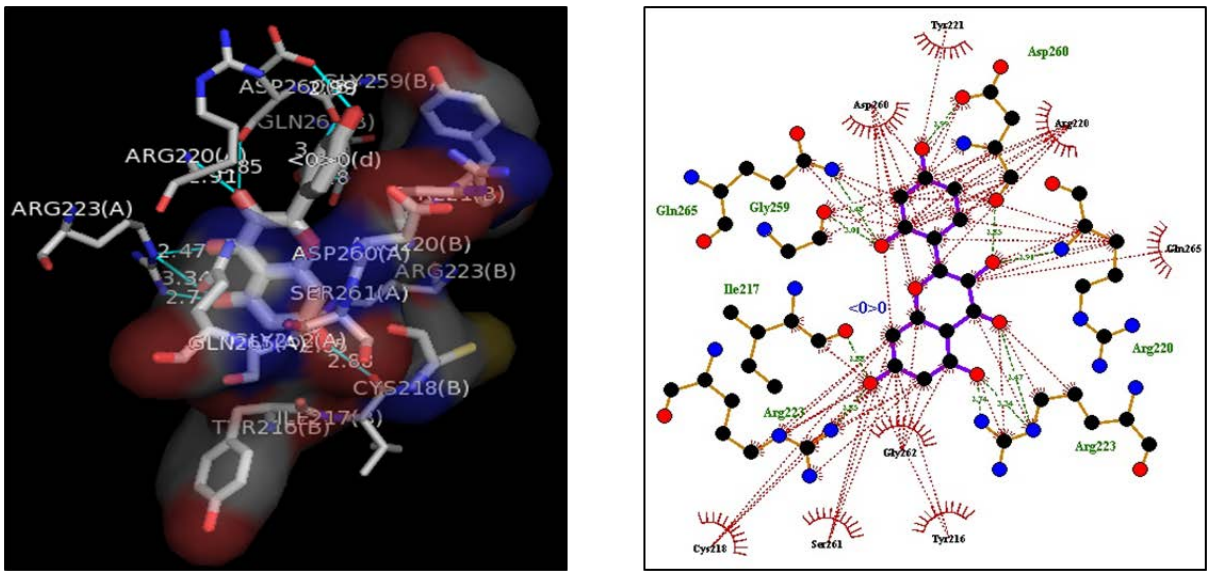


Figure 9b: 3D and 2D interaction between Morin and GSK3β.

GSK3β interaction with Myricetin

The interaction of GSK3β with Myricetin is shown in Figure 10a. 3D and 2D interaction using LIGPLOT+ is shown in Figure 10b. The minimum estimated free energy of binding calculated using autodock was -2.15 kcal/mol. The most common residues interacting between Myricetin and GSK3β are SER^{261}, GLN^{265}, ASP^{252}, ARG^{220}, TYR^{221}, ARG^{223}, CYS^{218}, ASP^{260} and GLY^{259}.

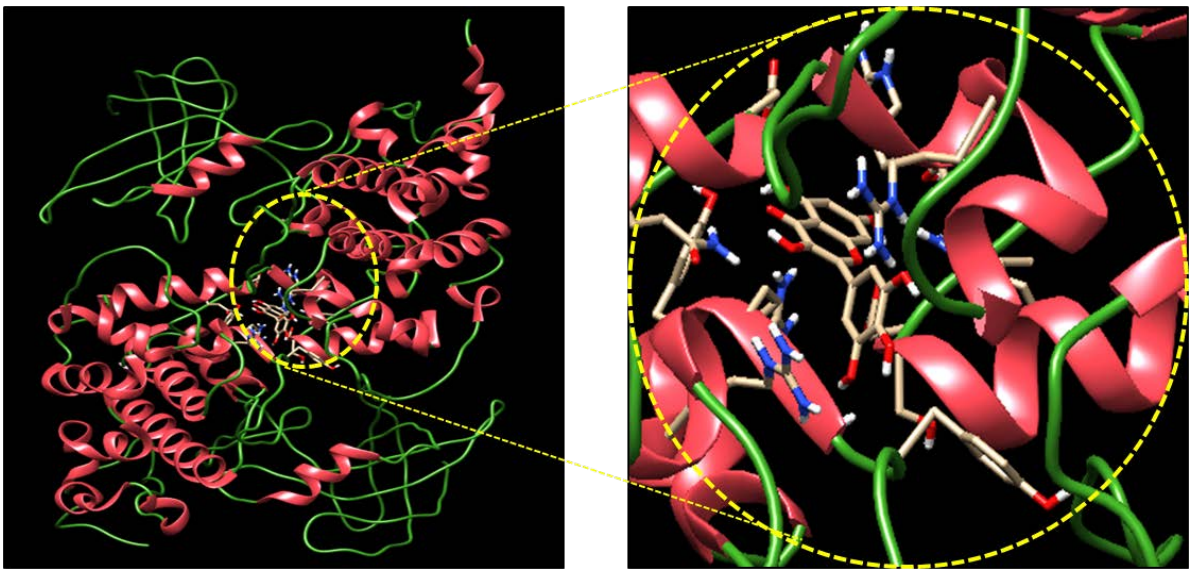


Figure 10a:GSK3β interaction with Myricetin

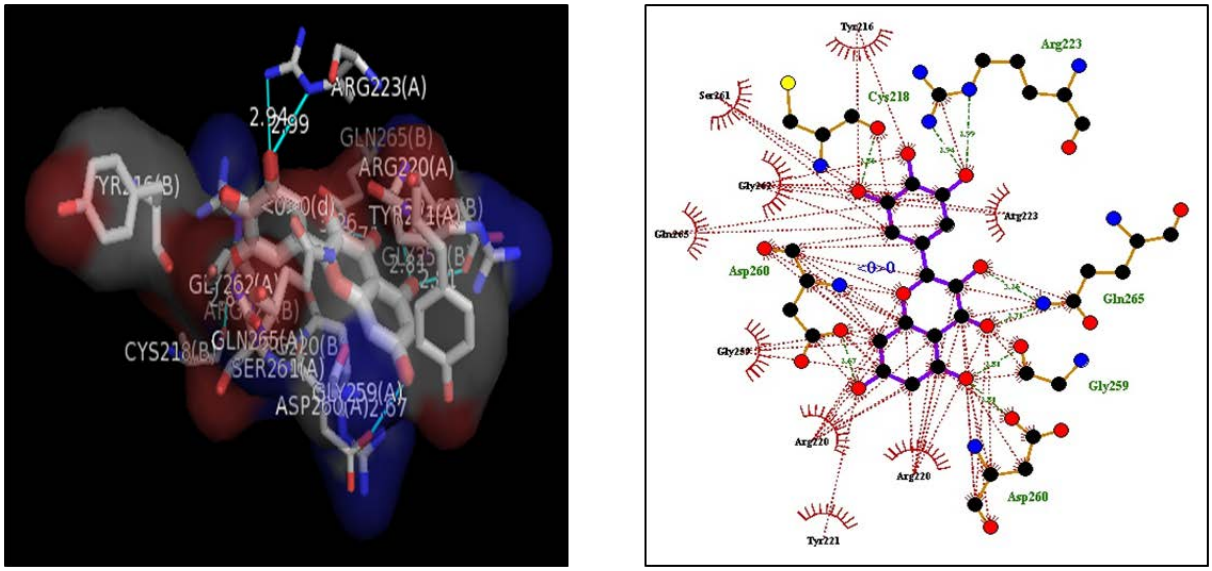


Figure 10b: 3D and 2D interaction between Myricetin and GSK3β.

GSK3β interaction with Quercetin

The interaction of GSK3β with Quercetin is shown in Figure 11a. 3D and 2D interaction using LIGPLOT+ is shown in Figure 11b. The minimum estimated free energy of binding calculated using autodock was -2.33 kcal/mol. The most common residues interacting between Quercetin and GSK3β are TYR^{216}, GLN^{265}, ASP^{260}, TYR^{221}, GLY^{262}, SER^{261}, ARG^{220}, ILE^{228}, GLY^{259}, GLY^{265} and ARG^{223}.

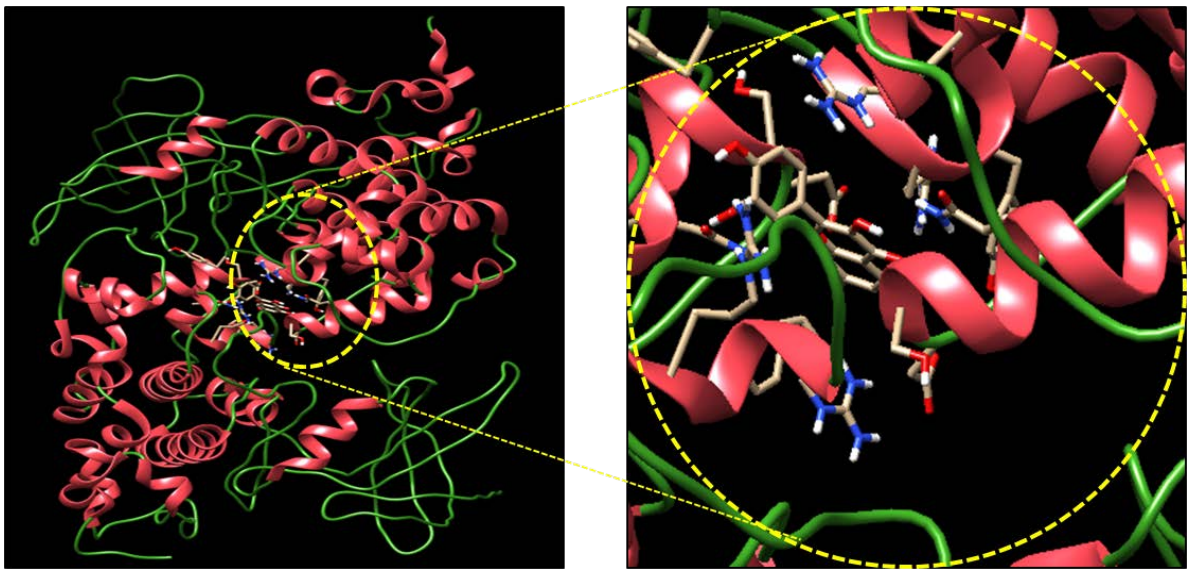


Figure 11a:-GSK3β interaction with Quercetin

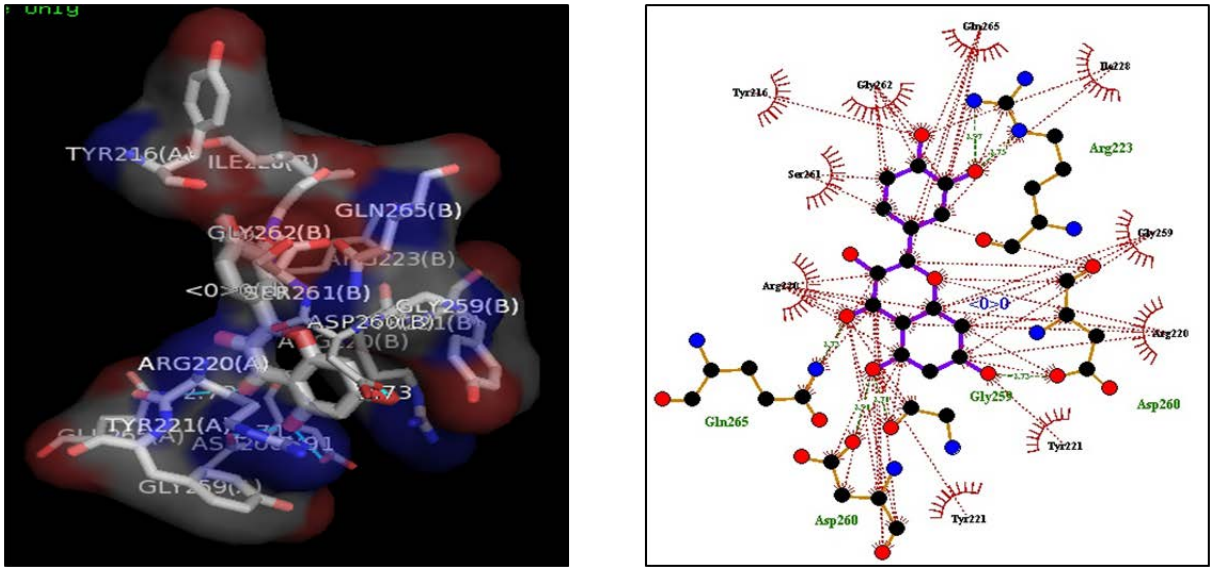


Figure 11b: 3D and 2D interaction between Quercetin and GSK3β.

CDK5 interaction with Kenpaullone

The interaction of CDK5 with Kenpaullone is shown in Figure 12a. 3D and 2D interaction using LIGPLOT+ is shown Figure 12b. The minimum estimated free energy of binding calculated using autodock was -3.25 kcal/mol. The most common residues interacting between Kenpaullone and CDK5 are ASP^{144}, VAL^{18}, ILE^{30}, THR^{17}, TYR^{15} and GLY^{16}.

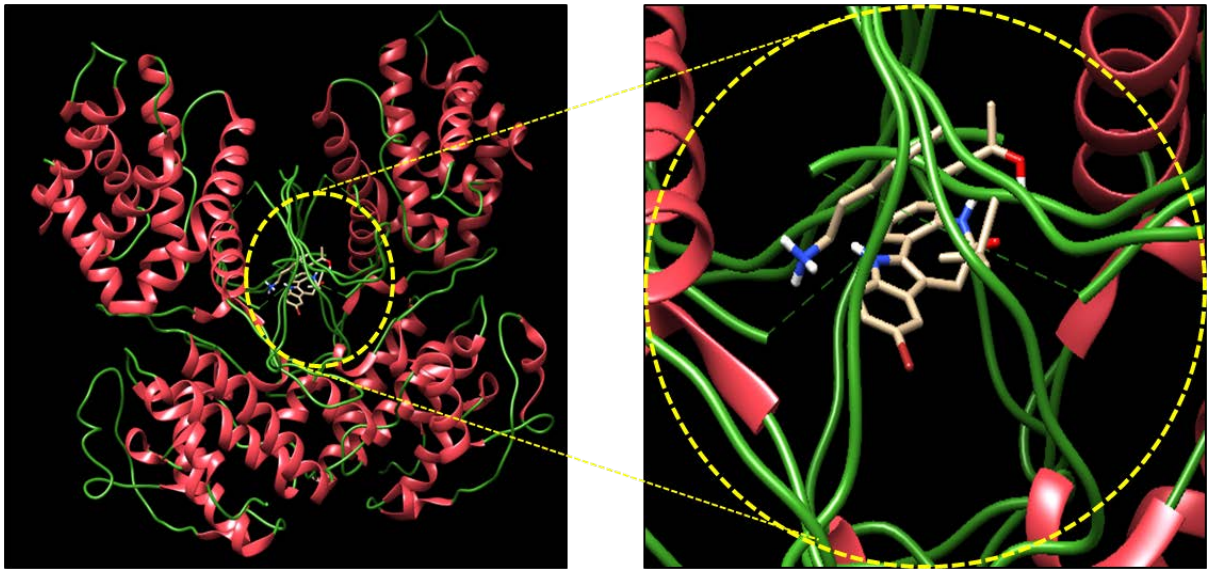


Figure 12a:CDK5 interaction with Kenpaullone

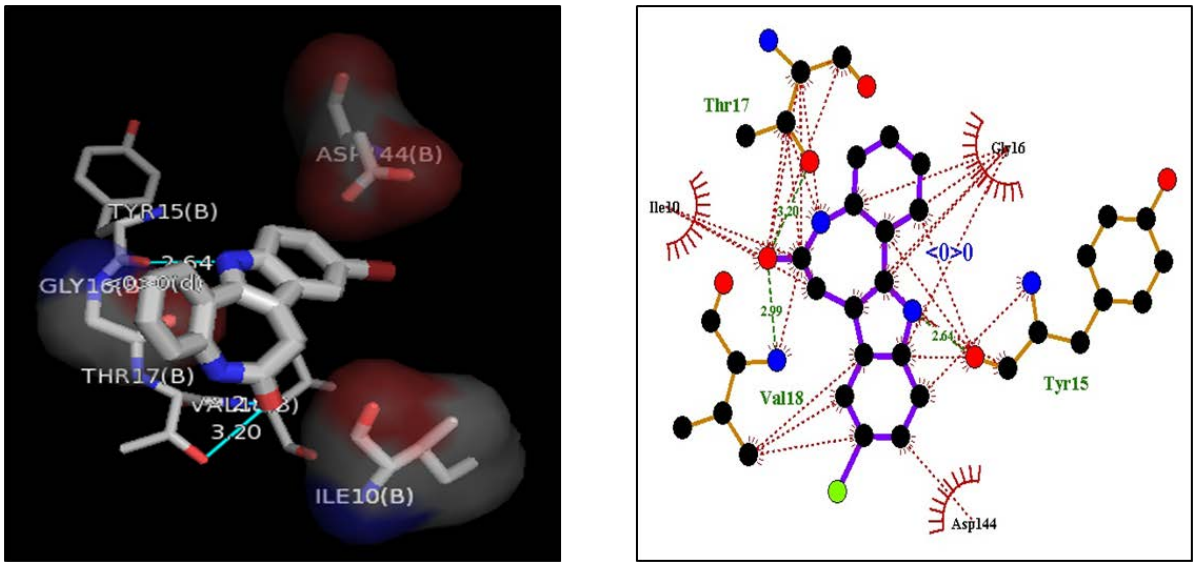


Figure 12b: 3D and 2D interaction between Kenpaullone and CDK5.

CDK5 interaction with Luteolin

The interaction of CDK5 with Luteolin is shown in Figure 13a. 3D and 2D interaction using LIGPLOT+ is shown in Figure 13b. The minimum estimated free energy of binding calculated using autodock was -3.03 kcal/mol. The most common residues interacting between Luteolin and CDK5 are VAL[18], THR[17], GLY[16], ILE[10] and TYR[15].

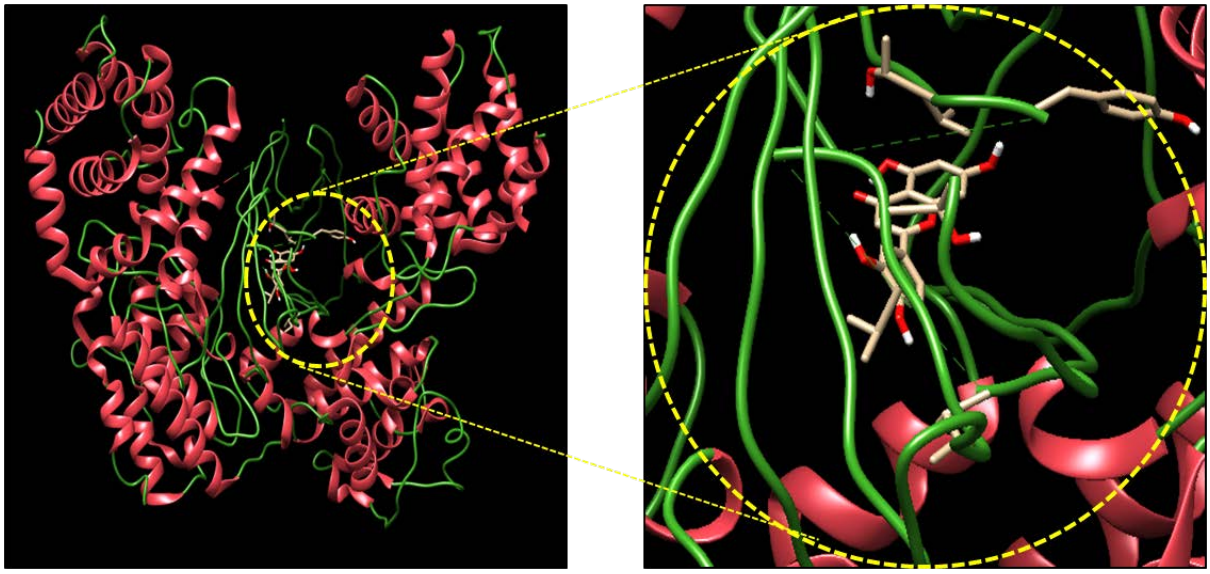


Figure 13a:CDK5 interaction with Luteolin

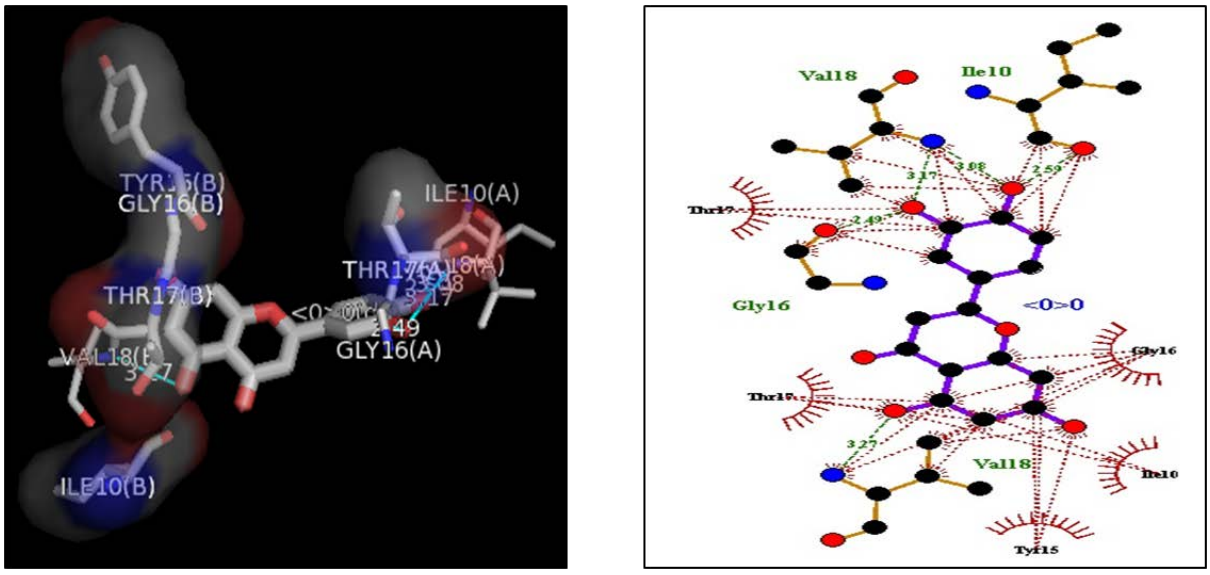


Figure 13b: 3D and 2D interaction between Luteolin and CDK5.

CDK5 interaction with Morin

The interaction of CDK5 with Morin is shown in Figure 14a. 3D and 2D interaction using LIGPLOT+ is shown in Figure 14b. The minimum estimated free energy of binding calculated using autodock was -2.45 kcal/mol. The most common residues interacting between Morin and CDK5 are TYR^{15}, LYS^{9}, GLY^{16}, VAL^{18}, THR^{17} and ILE^{10}.

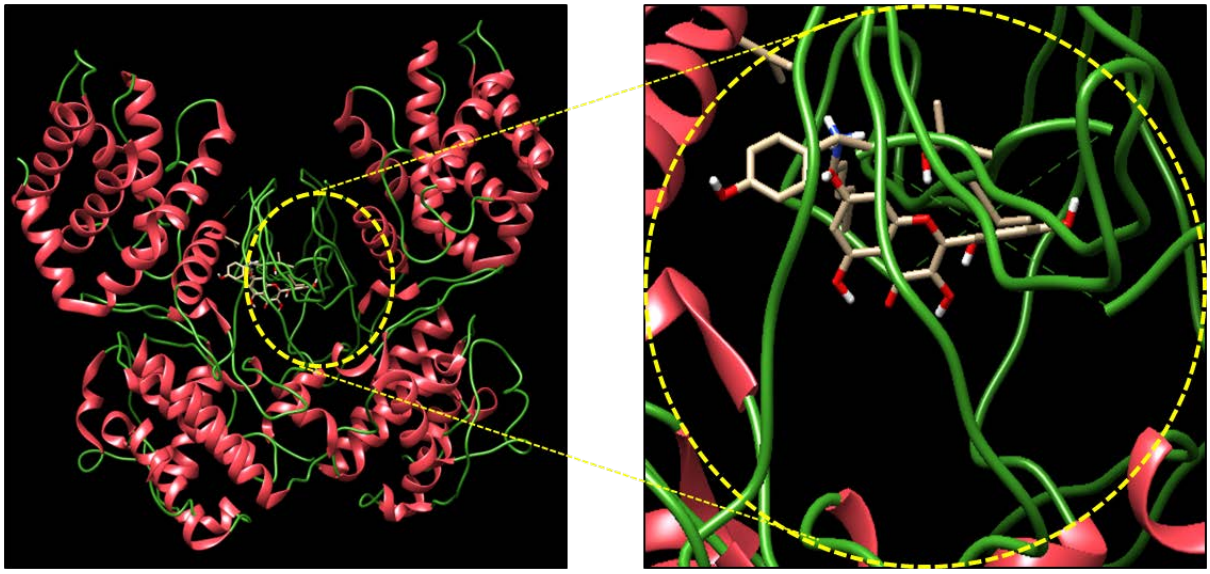


Figure 14a:CDK5 interaction with Morin

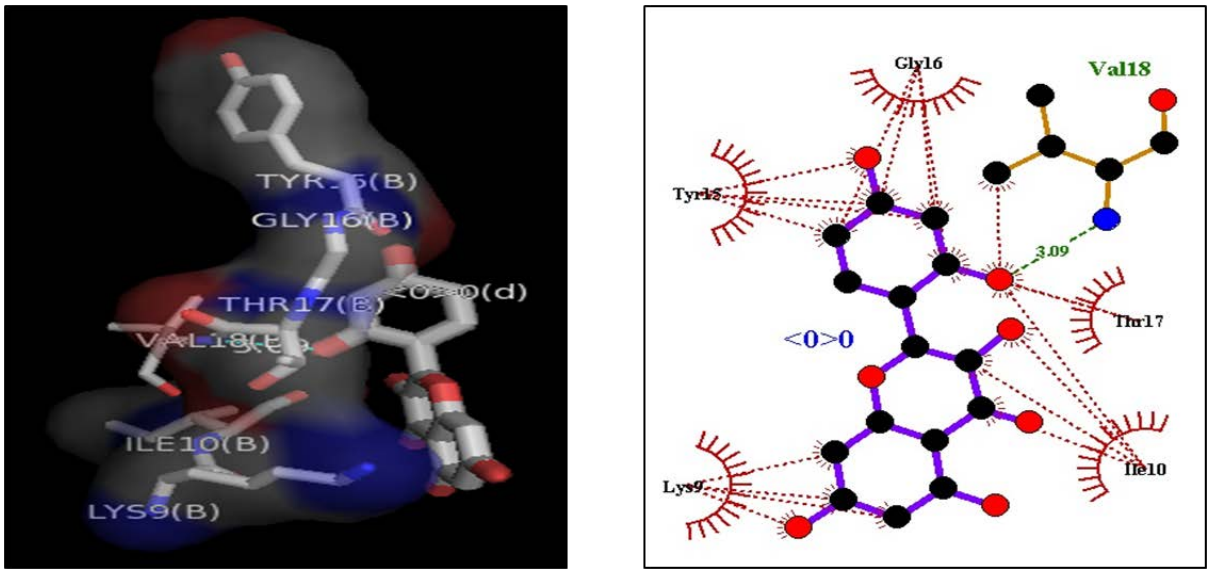


Figure 14b: 3D and 2D interaction between Morin and CDK5.

CDK5 interaction with Myricetin

The interaction of CDK5 with Myricetin is shown in Figure 15a. 3D and 2D interaction using LIGPLOT+ is shown in Figure 15b. The minimum estimated free energy of binding calculated using autodock was -2.36 kcal/mol. The most common residues interacting between Myricetin and CDK5 are LYS^{9}, TYR^{15}, ILE^{10}, VAL^{18}, THR^{17} and GLY^{16}.

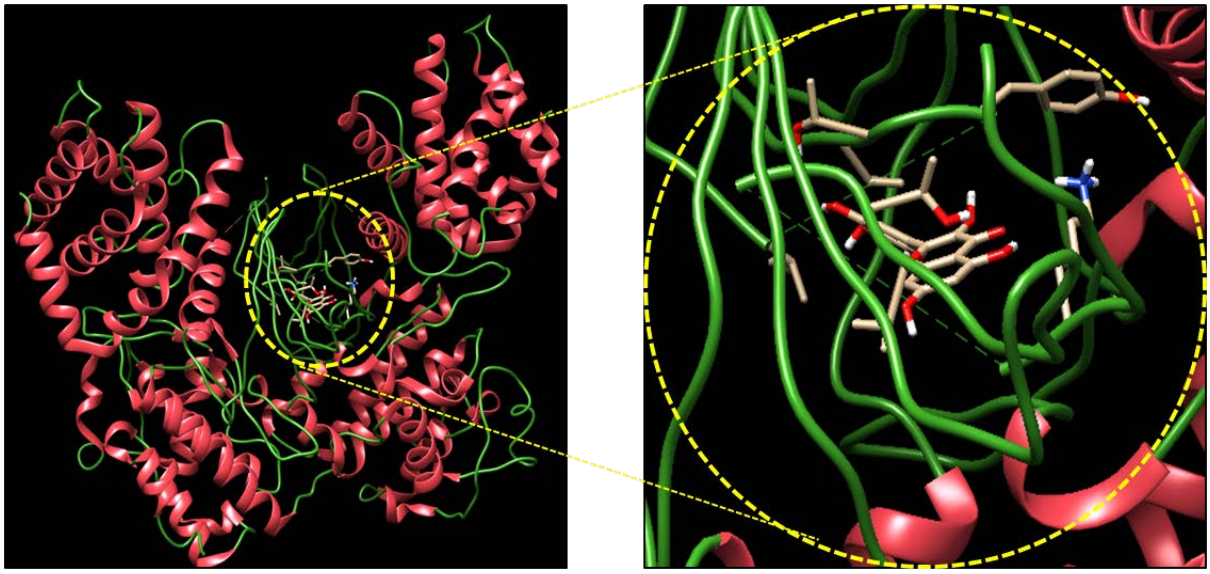


Figure 15a: CDK5 interaction with Myricetin

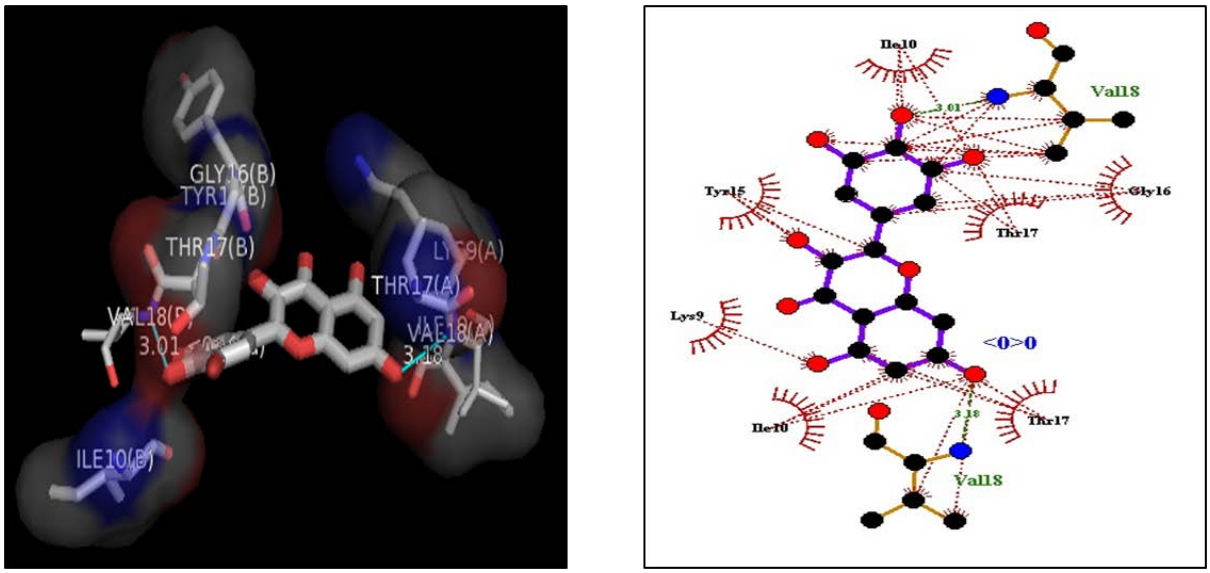


Figure 15b: 3D and 2D interaction between Myricetin and CDK5.

CDK5 interaction with Quercetin

The interaction of CDK5 with Quercetin is shown in Figure 16a. 3D and 2D interaction using LIGPLOT+ is shown in Figure 16b. The minimum estimated free energy of binding calculated using autodock was -1.30 kcal/mol. The most common residues interacting between Quercetin and CDK5 are ILE[10], THR[17], ILE10, VAL[18], GLY[16] and TYR[15].

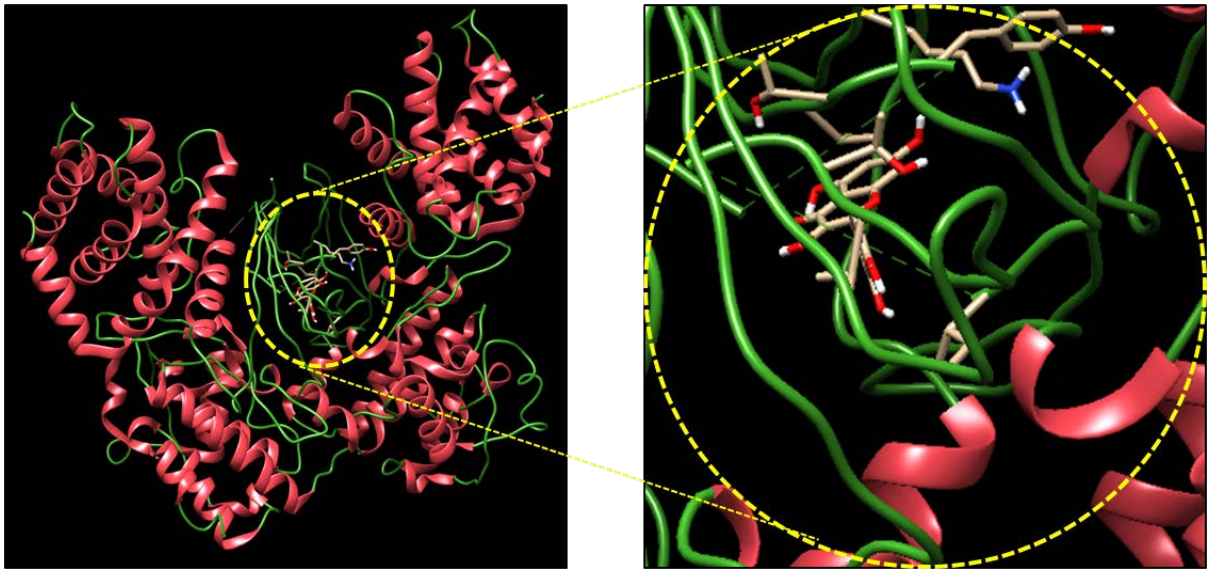


Figure 16a:CDK5 interaction with Quercetin

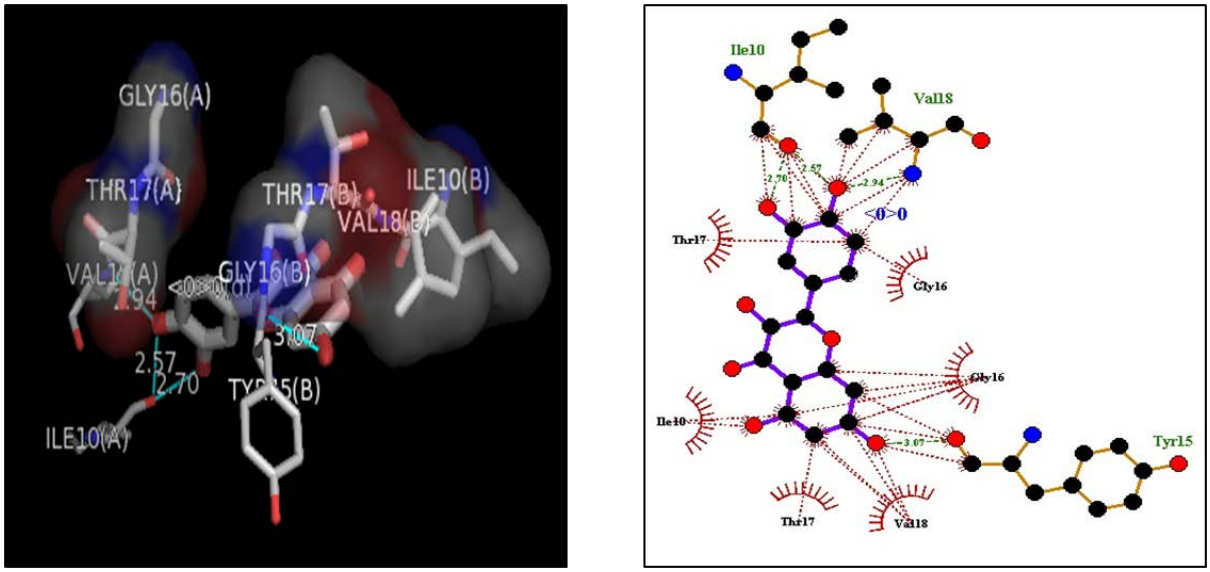


Figure 16b: 3D and 2D interaction between Qurecetin and CDK5.

CHAPTER 5: CONCLUSION

Tau is a microtubule associated protein and plays a crucial role in Alzheimer's as its hyperphosphorylation leads to dissociation from microtubule and formation of neurofibrillary tangles. Among many therapeutic approaches to treatment are drugs which inhibit kinases involved in hyperphosphoryation of Tau. *Insilico* docking was performed to find the inhibiting capacity of natural plant derivatives against tau protein kinase enzymes GSK3β and CDK5. Four different plant derivatives were taken for *insilico* docking studies; one known inhibitor of both enzymes was taken as control. AutoDock 4.2 was used for this purpose. Target/ligand complex models formed after docking were analysed on the basis of various parameters such as binding energy, hydrogen bond interactions, inhibition constant etc. The most common amino acid residues which are interacting with ligands in GSK3β are SER^{261}, ARG^{223}, GLY^{262}, GLY^{259}, Arg^{220}, Tyr^{216} and ASP^{260}. While VAL^{18}, THR^{17}, TYR^{15} and GLY^{16} were the most interacting residues in CDK5. These amino acids may be crucial in developing a suitable inhibitor. Among the selected flavonoids, Luteolin displayed the lowest binding energy comparable to the control Kenpaullone. The minimum estimated free energy of binding of Luteolin was -3.83 kcal/mol for GSK3β and -3.03 kcal/mol for CDK5. All selected flavonoids meet the Lipinski's filter requirement for a Drug. *Invivo* study of effects of drugs can help validate the results.

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