IDENTIFICATION OF NATURAL INHIBITORS OF PROTEINS INVOLVED IN PATHOLOGY OF PARKINSON'S DISEASE

Project report submitted to the Central University of Punjab

For the award of

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In

Biochemistry and Microbial Sciences

By

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May, 2018

CERTIFICATE

I declare that the project work entitled “Identification of natural inhibitors of proteins involved in the pathology of Parkinson’s disease." has been prepared by me under the guidance of Dr. Shashank Kumar, Assistant Professor, Department of Biochemistry and Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this project work has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Prareeta Mahapatra has prepared her project work entitled “Identification of natural inhibitors of proteins involved in the pathology of Parkinson’s disease ", for the award of M.Sc. degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department of Biochemistry And Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab.

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ABSTRACT

Identification of natural inhibitors of proteins involved in the pathology of Parkinson's disease

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Parkinson's disease (PD) is a progressive neurodegenerative disorder caused due to the lack of dopamine in the brain. Different drug therapies are available for PD showing excellent efficiency, but most of them are cost intensive and with side effects. All these issues have brought natural products in attention. The present study was designed to identify the potent anti-Parkinson phytochemicals. Proteins that are involved in Parkinson's disease were targeted. In the present study, methylated flavonoids were selected for studies including molecular docking against protein involved in Parkinson's disease such as Murine Keap 1(5CGJ), brain permeable Polo-like kinase (4I5P), Methionyl tRNA synthetase(1PFU) and Roco-4-kinase(4F0F).To predict the drug-likeness property of the phytochemicals, Lipinski's rules of five, Caco-2, CMC-like rule and MDCK value were used. By prediction of ADME, drug-likeness properties and toxicity properties of the phytochemicals it can be stated that most of the phytochemicals have the potential to cross the Blood Brain Barrier and have good ROS quenching potential also.

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Prareeta Mahapatra

Date

LIST OF ABBREVIATIONS

Abbreviations	Expansion
6-ODHA	6-hydroxydopamine
ADMET	Absorption, distribution, metabolism, excretion and toxicity
ARE	antioxidant responsive element
AT	Ames test
BBB	Blood-brain barrier
CADD	Computer-aided drug designing
CLR	CMC like rule
CR	Carcino rat
DA	Dopamine
DJ-1	Protein deglycase
DNA	Deoxyribonucleic acid
FDA	Food and drug administration
GABA	Gamma aminobutyric acid
GCLC	Glutamate-cysteine ligase catalytic subunit
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
hERG-I	Human Ether-ά- go- go gene inhibition
HIA	Human Intestinal Absorption
LRRK2	Leucine-rich repeat kinase 2
M	Mutagen
MAO-B	Monoamine oxidase
MDCK	Madin-Darby Canine Kidney cells
MPP^+	1-methyl-4-phenylpyridinium
MPT pore	mitochondrial permeability transition pore
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NM	Non-mutagen
NQ	Not qualified
Nrf-2	nuclear factor erythroid 2–related factor 2
PD	Parkinson's Disease
P-gp I	P-glycoprotein inhibition
PINK2	PTEN (phosphate and tensin homolog)-induced putative kinase 1
PLK	Polo-like Kinase
PPB	Plasma Protein Binding
Q	Qualified
RF	Rule of five
ROS	Reactive oxygen species
SNCA	Synuclein alpha
tRNA	Transfer RNA

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1.1 Parkinson's disease and natural products

Parkinson's disease (PD) is the second most prevalent progressive neurodegenerative disorder which is caused due to the lack of dopamine in the brain and is characterized by tremor, akinesia, bradykinesia and postural instability. Mutations in different genes e.g. SNCA, LRRK2, Parkin, PINK2, DJ-1, etc. are found to be involved in PD and these genes can be targeted to treat PD up to an extent (Elbal, *et al,* 2002). Different drug therapies are available for PD, showing excellent efficiency, but most of them are cost-intensive, with side effects and can only cure the disease symptomatically (only reduction in symptoms). All these issues have brought natural products in attention. Since last decades various phytochemicals are actively used by people to treat many kinds of diseases. Natural products derived from plants have made their own position in the treatment of different neurological disorders. Proper considerations and clinical trials were made on some phytochemicals and clinical tests were made for their validation, mechanism of working and biochemical recovery. Plant-derived molecules have already been examined for their cellular, behavioral and biochemical protection against PD and many other neurological disorders. These phytochemicals are easily available and have no/very poor side effects. Moreover, these also have antioxidant properties. For example terpenoids like geraniol, catalpol, tanshinone and flavonoids like baicalein and lactones like EGCG etc. are on clinical trials and are proving as potential natural products that can inhibit different causes that lead to PD (Suk, *et al,* 2005). Natural products have played a very crucial role in drug discovery and have led to a revolution in pharmacology and medicine. Many natural anti-Parkinson drugs have been approved internationally and actively used in its therapy. Many other natural phytochemicals are under clinical trials. New approaches like Computer-aided drug designing (CADD) is being exploited to identify hits, pick leads and optimize drug leads by studying their physicochemical, pharmaceutical and ADMET (absorption, distribution, metabolism, excretion and toxicity) properties for more precise validation of these natural products as antiparkinson drugs. These new approaches can play a critical role in the treatment of PD by identification of leading phytochemicals that can inhibit different proteins involved in the pathology of Parkinson's disease.

1.2 Objective

The objective of the present study is to identify the active phytochemicals against Parkinson's as well as their scientific validation using *in silico* studies.

2.1 Parkinson's Disease

Parkinson's disease is a progressive neurodegenerative disorder which is caused due to the lack of dopamine in the brain and is characterized by tremor, akinesia, bradykinesia and postural instability. The main cause of the disease was discovered to be the fall in the level of dopamine in the brain. Death of dopaminergic neurons in the substantia nigra of the brain leads to decrease in the level of dopamine which in turn results in overstimulation of acetylcholine target neurons. More Acetylcholine induces overstimulation of GABAergic neurons in the substantia nigra and then in the thalamus. As a result, the balance between glutamate and GABA is disrupted which results in abnormal signaling and leads to impaired mobility. Different causes of death of dopaminergic neurons include accumulation of alpha-synuclein fibrils in the substantia nigra part of the brain due to the mutation in the SNCA gene or alpha-synuclein gene (Polymeropoulos, *et al*, 1997), which leads to proteasomal and lysosomal system dysfunction and reduced mitochondrial activity by affecting its complex I (Keeney, *et al*, 2006), LRRK2 gene codes for leucine-rich repeat kinase is the only known gene coding for a protein having both kinase and GTPase domain. Mutation in this gene is responsible for the decrease in the GTPase activity and impaired kinase activity which leads to dysfunctioning of presynaptic protein sorting and axonal trafficking, Parkin gene, a part of ubiquitin-ligase complex when homozygously mutated, disrupts the activity of the later which leads to the accumulation of toxic substrates. It leads to the formation of Lewy bodies and finally death of dopaminergic neurons occurs. Other genes involved in PD are DJ-1, PINK-1, transcription factor Nrf-2 etc. (Elbal, *et al*, 2002). All these proteins are targeted either by synthetic drugs or by drugs obtained from natural products.

2.2 Natural antiparkinson products

Natural products have been a great source for treatment of PD. They provide a significant approach on where and how to inhibit different molecular and biochemical causes of PD. The antioxidant medicinal plants are a good source for protection against Parkinson. These include Brahmi that may improve brain circulation and even protect brain cells from further damage, Cowhage contains

Levodopa or L-dopa is administered against Parkinson's disease. (Szego, *et al,* 2012) Turmeric contains a compound curcumin that is responsible for the disruption of proteins involved in PD and it also prevents their aggregation, *Gingko Biloba* extract showed neuroprotective and neuro-recovery effects against dopaminergic neuron damage and even damage that affects locomotion.

2.3 History of natural antiparkinson products

The concept of natural products has its roots back in 17th and 18th century. Many plant products are used since those days and have been established as potential drugs against PD. Epicatechin-3-gallate (EGCG) in *Camelia sinensis* (green tea), is the most abundant polyphenol and has anti-inflammatory and neurodegenerative effects. The root extract of *Withania somnifera* is rich in steroidal lactones (withanone, withaferin, withanolides, and withasomidienone). These were discovered to have potential to inhibit metastasis and Quinone reductase activity. Caffeine, an adenosine 2A receptor antagonist present in coffee beans, inhibits the MPTP induced toxicity by depleting the toxic product 6-ODHA. Ginkgolides and Bilobalides found in *Ginkgo biloba* have a protective role against ROS production (Smith, *et al*, 1996) and oxidative stress (DeFeudis, *et al*, 2000). Baicalein, a flavonoid found in large concentration in *Scutellaria baicalensis*, attenuates the iron-induced DA–depletion in the substantia nigra and also inhibits the alpha-synuclein aggregation by increasing GSH level (Hamada, *et al*, 2002). Levodopa was first isolated from the leguminous plant *Mucuna pruriens* (velvet beans) is an FDA approved drug used for the treatment of PD. Historically natural products have been a rich source of compounds that have a great importance in medicines and therapeutics. Drugs of natural origin can be classified as original natural products, products derived semi-synthetically from natural products or synthetic products based on natural product models. Natural products were examined to have a significant effect on the pathology of Parkinson's disease. The knowledge associated with traditional medicine and use of medicinal plants as potential medicines has led to the isolation of many natural products that have become well-known pharmaceuticals today.

2.4 Importance and advantages of natural antiparkinson products

Due to development of resistance to therapeutic drugs search for new antiparkinson drugs and therapies is still a priority goal for the treatment of PD. Natural products considered to be the best source of drugs for past many years as these having no or very poor side-effects, easy availability, and cost-effective. Moreover, natural products have structural and chemical diversity, and there is a substitute option most of the time when one is not available. Hence, natural products are a good source of active therapeutic agents.

2.5 Currently used natural antiparkinson products

Several phytochemicals are known to treat or manage PD. Some are discussed in the next section.

2.5.1 Crocin

Crocin, a carotenoid found in flowers, is primarily responsible for the color of saffron. It acts as an antioxidant, as it quenches free radicals, protects cells and tissues against oxidation. Pretreatment of cells with crocin inhibits ROS generation in the dopaminergic terminal and hence prevents the death of dopaminergic neurons. (Zhang, *et al*, 2015).

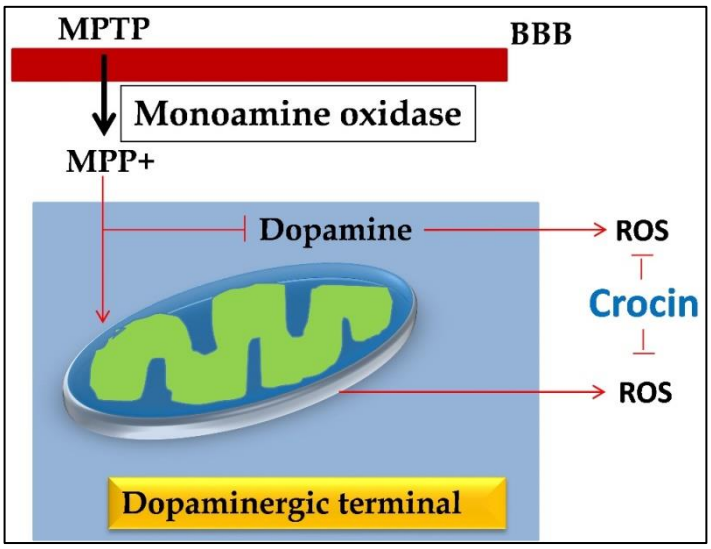

Figure 2.1: Inhibition of ROS by Crocin

2.5.2 Geraniol

Geraniol is a monoterpenoid and alcohol. It is the primary part of palmarosa oil, rose oil, citronella oil, lemon and much other essential oils (Tiwari, *et al*, 2009). It acts as an antioxidant and is able to cross the blood-brain barrier. It has cytoprotective characteristics against oxidative stress produced due to various neurotoxins. It helps to restore the membrane potential of mitochondria. Case 1: In the blood-brain barrier, monoamine oxidase-B is present which is responsible for converting non-toxic MPTP to toxic MPP^+ (Rekha, *et al*, 2013). The administration of geraniol in the early stage of PD can destabilize the enzyme MAO-B, and hence toxin formation can be put to an end (Ben-Shlomo, *et al*, 2004). Case 2: Geraniol can prevent the aggregation of alpha-synuclein and hence prevents the formation of alpha-synuclein inclusions, which is a major cause of PD and other neurological disorders. (Szego, *et al*, 2012)

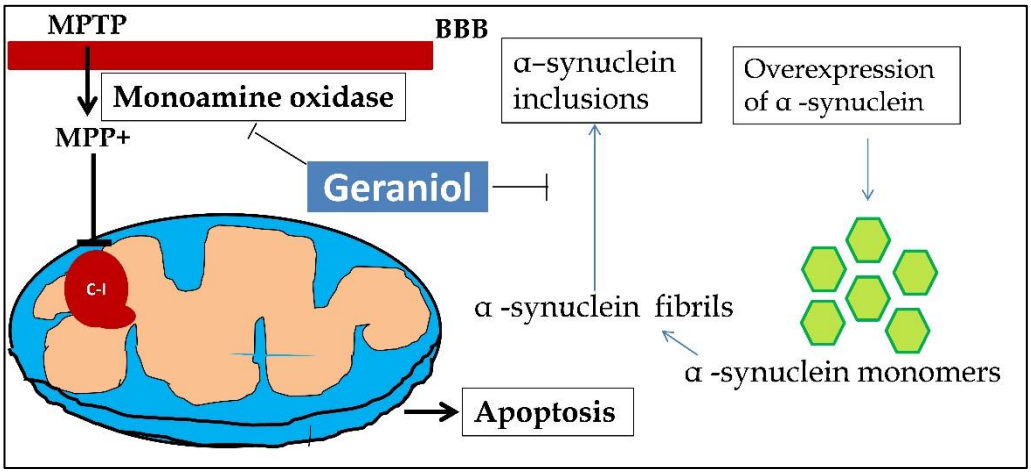

Figure 2.2*:* Geraniol inhibits MAO-B and α-synuclein aggregation

2.5.3 Safranal

Safranal, an organic compound isolated from saffron, is a spice that contains the stigma of crocus flower which is completely responsible for saffron's aroma. It inhibits rotenone-induced cell death (Radad, *et* al, 2006). Rotenone significantly increases the release of lactate dehydrogenase into the surrounding. It decreases the membrane potential of mitochondria and

increases the production of reactive oxygen species, and an anaerobic state of respiration is established (Pan, *et al*, 2016).

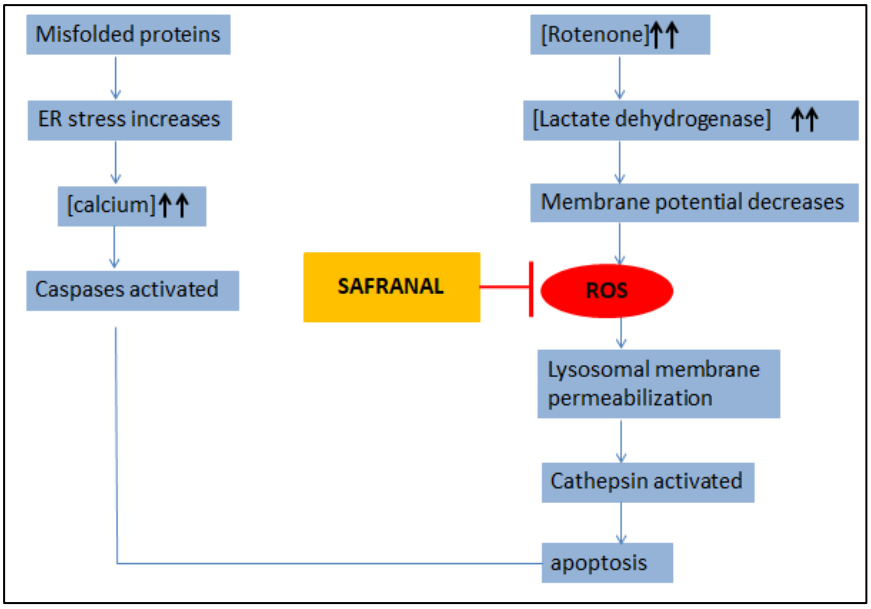

Figure 2.3: Safranal inhibits ROS production

2.5.4 Catalpol

Catalpol is an iridoid glycosides, simple monoterpenes with an attached glucose moiety. It is found in the plants that belong to families like Scrophulariaceae, Lamiaceae, and Bignoniaceae, etc. MPP+ is found to be responsible for the opening of (Mitochondrial permeability transition) MPT pore and release of cytochrome c in brain and mitochondria (Iverson, *et al*, 2004). This is responsible for the mitochondrial destruction (Mao, *et al*, 2007). Catalpol treatment reduces the activity of MPP^+ regarding the opening of MPT pore (Bi, *et al*, 2008). On the other hand, Ca^{2+} concentration is also elevated in case of neuronal cells of PD patients, which is also responsible for the opening of MPT pore. Catalpol also restrains the pressure of overloading Ca^{2+}.

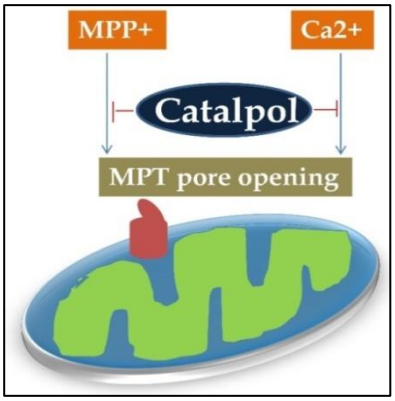

Figure 2.4: Catalpol inhibiting trigger compounds for MPT pore opening.

2.5.5 Tanshinone

Tanshinone is a diterpenoid and a major lipophilic bioactive compound found in *Salvia miltiorrhiza* (Family I). It mediates neuroprotective activity against 6-OHDA-induced oxidative stress via an Nrf2-Are pathway (Kaidery, *et al*, 2013). The transcription factor Nrf2 plays an important role in the induction of cytoprotective genes like those that encode for endogenous antioxidants such as heme oxygenase-1, glutathione cysteine ligase regulatory subunit (GCLC) and glutathione cysteine ligase modulatory subunit (Todorovic, *et al*, 2016). On the other hand, 6-OHDA is a neurotoxin that is initiated by extracellular auto-oxidation of oxidative products that are generated (Hanrott, *et al*, 2006). The loss of Nrf2-mediated transcription increases the chances of dopaminergic neurons to undergo oxidative stress (Jakel, *et al*, 2007). Normally, Nrf2 is regulated post-translationally and constitutively by Keap1, its antagonist (Jing, et al, 2016). The actual function of Nrf2 begins when the cell is exposed to oxidative stress or electrophilic substances. As a result, the Keap1 is modified, detaches from Nrf2, and proceeds into the nucleus and transactivates many target genes. Under resting metabolic conditions, negative regulation of Nrf2 is mediated by Keap1 through ubiquitination. Under conditions of stress, Keap1 is oxidized, and Nrf2 is released, which is stabilized by DJ-1 and translocates to the nucleus via ARE enhancers do activates a range of antioxidant enzymes and hence decreases the oxidative stress (Pan, *et al*, 2016)

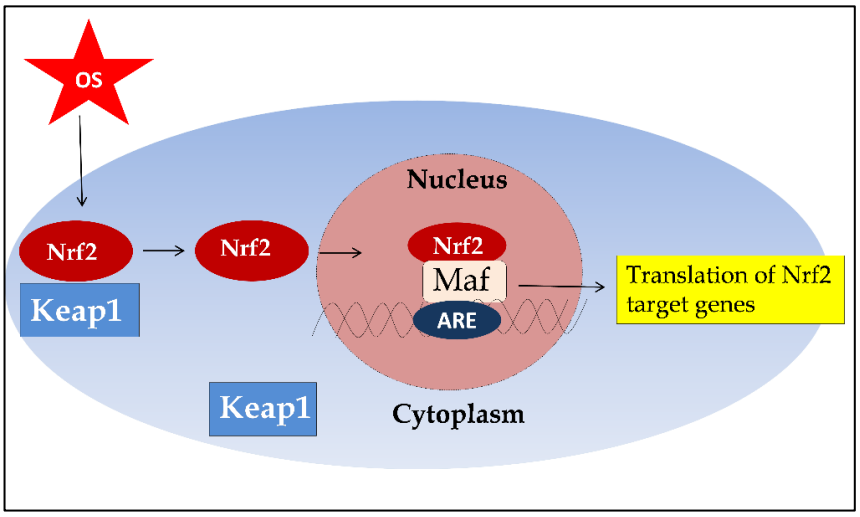

Figure 2.5: Nrf2 released by Keap1, Nrf2 enters nucleus for translation

3.1 *In silico,* antiparkinson potential screening

3.1.1 Protein preparation

Crystal structure of target protein, such as Methionyl tRNA synthetase (1PFU), Roco-4 kinase (4F0F), selective and brain-permeable polo-like kinase 2 (415P), murine keap1 (5CGJ), and were retrieved from Protein Data Bank. All the heteroatoms were removed leaving only the residues of the receptor. Preparation of the target protein with Auto Docking Tool involved the addition of polar hydrogen to the macromolecule, to correct the calculation of partial charge. Finally, Gasteiger charges were calculated for each atom of the macromolecule.

3.1.2 Ligand preparation

Literature-based phytochemicals have been used as a ligand for molecular docking. The 3D or 2D structure of phytochemicals and reported inhibitors of particular protein were retrieved from NCBI PubChem in sdf format respective. Open Babel molecule format converter was used for conversion of 2D to 3D conformation, Marvin Sketch software will perform the conversion from sdf to PDB (for docking) and mol (for molecular properties prediction) file. The energy of the ligands was minimized by applying mmff94 force field and optimization of conjugate gradients algorithm was done using PyRx-Python prescription 0.8 (Olson, *et al*, 2015) for 200 steps.

3.1.2.1 Molecular docking

For the docking of different targeted proteins with selected ligands (here the ligands are methylated flavonoids) virtual molecular screening is used. Docking of the ligands and proteins was done in Pyrx software. In Pyrx software, the proteins were first loaded and then converted to macromolecule. The ligand (one at a time) is imported and their energy was minimized by application of mmff94 force field. Then axes, conformations and orientations of the ligands were set in the protein structure by clicking on the vina wizard. Then it was run to obtain the binding affinity (table 4.3). Docking was performed with the targeted protein interface by keeping the points of X, Y and Z dimensions as 25.0000, 25.0000 and 25. 0000 respectively and center grid box values were kept 15.066, 54.587 and 13.5861 for x, y and z centre of Methionyl-tRNA synthetase; -6.6117, 20.2038 and -18.5522 for

x, y and z of Roco-4 kinase; 10.2022, 7.0789 and 10.2418 for x, y and z of polo-like kinase; 40.06, -20.46 and -4.9 for x, y and z of Murine keap-1. The grid boxes represent the entire binding site of the protein interface and provide space for the ligand's binding with respect to all the three axes. Then visualization of the interaction pattern of the complex then formed was done (the protein-ligand complex).

3.1.2.2 ADME Prediction

ADME is used as an abbreviation for Absorption, Distribution, Metabolism and Excretion in pharmacology. These four criteria influence the drug levels and drug exposure to tissue and influence the kinetics related to the drug's performance and pharmacological activity of a compound as a drug. Blood Brain Barrier (BBB), colorectal adenocarcinoma cells (Caco-2), Madin - Darby Canine Kidney cells (MDCK), HIA(Human Intestinal Absorption) and Plasma Protein Binding (PPB) properties were studied using pre - ADMET server.

3.1.3 Drug likeness prediction

Number of hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD), Mol Log P, Mol Log S, drug-likeness model score properties were studied using pre-ADMET server.

3.1.4 Toxicity prediction

AMES test, Human Ether-ά- go- go gene (HERG inhibition), Carcino rat properties were studied using pre- ADMET sever.

3.1.5 Ligplot analysis

Ligplot is the software that generates a 2D diagram showing ligand-protein interaction. It reveals the number of hydrogen bonds and hydrophobic interactions, as well as with which amino acids of the protein the ligand is interacting and the chain number also.

3.1.6 Surface structure analysis

In Ligplot 2D diagram, the ligand pocket or active site of the protein cannot be visualised, therefore Pymol was used to visualize the surface structures of the protein and ligand interaction.

3.2 In vitro screening Laboratory techniques:

3.2.1 Anticancer potential screening assay: MTT assay

To study the compound 005 cytotoxic effect on HT-29 colon cancer cell line, for this we seeded 10000 cells per well in 96 well plate in three in number. It was then incubated for 24hrs at 37^{o}C. After adhering on the plate, the cells were treated with different concentrations of drug for 24 hours. The cells were again incubated for 24 hrs, 48 hrs, 78hrs time intervals for identification of IC50 value of the drug. After incubation the media was removed and 100ul of MTT (5 mg/ml) was added. The cells were again incubated for 4 hrs and MTT was removed. Then 100µl of DMSO was added to each well. Then it was incubated for 5 minutes in dark and the plate was read at 590 nm in a spectrophotometer.

3.2.2 Antioxidant potential screening assay: DPPH assay

The free radical scavenging activity of the samples was measured *in vitro* by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Appropriate DPPH solution concentration was prepared in methanol followed by addition of 1 ml of the test sample at different concentration. The content was mixed and allowed to stand at measured by recording the absorbance at 517 nm. The percentage scavenging activities (%Inhibition) at different concentrations of the extracts were calculated using the following formula:

(%) I= [(Ac−As)/Ac] ×100

Where “I” is inhibition and Ac and As are the absorbance values of the control and the sample, respectively. Three replicates were made for each sample and results were expressed as mean ± SD.

3.2.3 Antibacterial potential screening assay: Disc Diffusion method

Antimicrobial activity of test samples against *Escherichia coli* was determined using Kirby-Bauer disc diffusion method. The inoculum suspensions of bacterial strains were swabbed on the entire surface of LB agar. Sterile 6 mm diameter paper discs (Himedia) saturated with 10μg of phytochemicals (drugs) prepared in

DMSO (containing 2 mg extract/disc) were aseptically placed on the upper layer of the inoculated agar surfaces and plates were incubated at 37°C for 24 hours. Antibacterial activity was determined by measuring the diameter of the zone of inhibition (ZOI) surrounding discs. Standard antibiotic discs of Penicillin and Norfloxacin were used as positive control. Discs containing 20 µL DMSO were used as a negative control. The antimicrobial assay was performed in triplicate and results were reported as the average of three replicates (Bauer, *et al*, 1996).

4.1 *In silico* antiparkinson's potential of identified phytochemicals

4.1.1 Molecular drug-likeness property and toxicity prediction of the phytochemicals

For the present study 69 phytochemicals (fig 4.1) were selected based on their suitability for Chemistry manufacture and control (CMC) like rule, Rule of Five. The Chemistry manufacture and control (CMC) like rule, Rule of Five distinguishes between the drug like and non-drug like molecules. These phytochemicals include 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-chromen-4-one, 5,7,8-trihydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-7-methoxy-4H-chromen-4-one, 2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-7-methoxy-4H-chromen-4-one, 8-hydroxy-7-methoxy-2-(2-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-2-(4-hydroxyphenyl)-7,8-dimethoxy-3-methyl-4H-chromen-4-one, 5-hydroxy-2-(4-hydroxyphenyl)-7,8-dimethoxy-3-methyl-4H-chromen-4-one, 3,5,6-trihydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5,6,7,8-tetrahydroxy-4H-chromen-4-one, 3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-8-methoxy-4H-chromen-4-one, 5,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-6,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 8-hydroxy-5,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-7,8-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-2-(4-hydroxyphenyl)-6,7,8-trimethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,6-trihydroxy-7-methoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,8-trihydroxy-7-methoxy-4H-chromen-4-one, 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-6,8-dimethoxy-4H-chromen-4-one, 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-6,8-dimethoxy-4H-chromen-4-one, 3,6,8-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-5,7-dimethoxy-4H-chromen-4-one, 5,6,7-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-7-hydroxy-5,6-dimethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-7-hydroxy-5,6-dimethoxy-4H-chromen-4-one, 3-hydroxy-2-(4-hydroxyphenyl)-5,6,7-trimethoxy-4H-chromen-4-one, 5-hydroxy-3,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-6,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(4-hydroxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one, 3-hydroxy-2-(4-hydroxyphenyl)-5,6,7-trimethoxy-4H-chromen-4-one, 5-hydroxy-6,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-2-(3-hydroxy-4-

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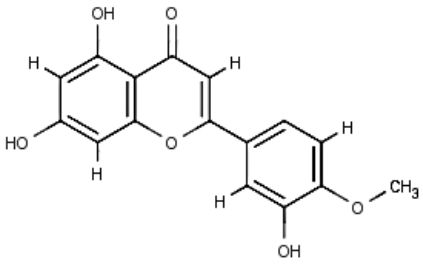
benzopyran-4-one, 2-(3,4-dimethylphenyl)-5,6,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-3,5,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-5,7-dimethyl-4H-chromen-4-one, (2S)-2-(3,4-dimethylphenyl)-5,6,7-trimethyl-3,4-dihydro-2H-1-benzopyran-4-one.

The ADME property, drug likeness property and toxicity property of the phytochemicals such as Blood brain barrier (BBB) penetration, Caco-2 permeability, Human Intestinal Absorption (HIA), Madin-Darby Canine Kidney cells (MDCK), Pgp inhibition, Plasma Binding Protein (PBP), CMC (Chemistry manufacture and control) like rule, Rule of five, mutagenicity (Ames test), Carcino Rat (CR) and hERG inhibition (hERG-I) were studied *in silico.*

Standard compounds N-(5-methyl-1H-pyrazol-3-yl)-2-phenylquinazolin-4-amine, 4-[4-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino]phenoxy]-N-methylpyridine-2-carboxamide, 2-methyl-5-propan-2-ylcyclohexa-2,5-diene-1,4-dione, 2-[[4-[carboxymethyl-(4-methoxyphenyl)sulfonylamino]naphthalen-1-yl]-(methoxyphe nyl)-sulfonyl- amino] acetic acid were used in the study to compare the results.



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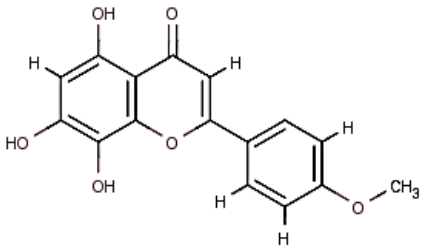

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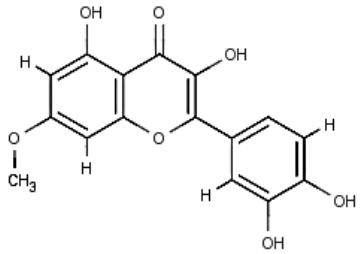

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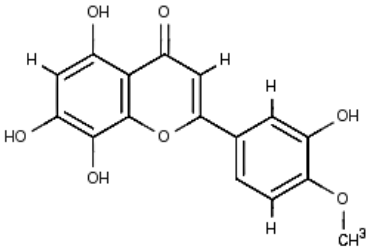

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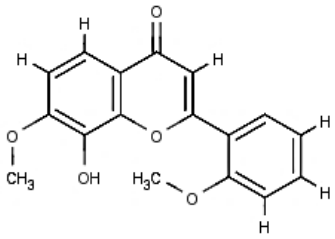

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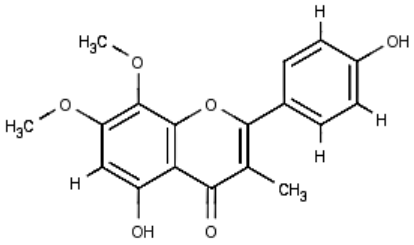

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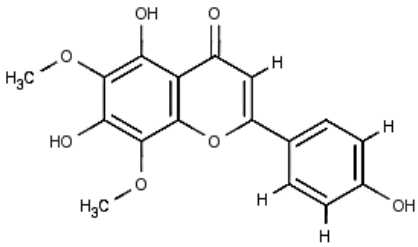

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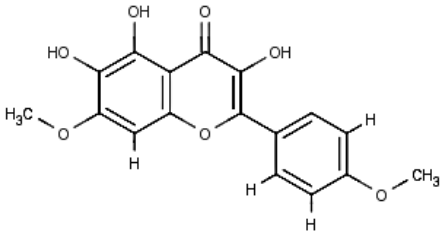

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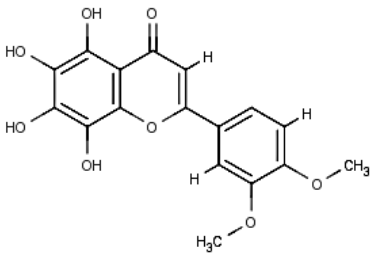

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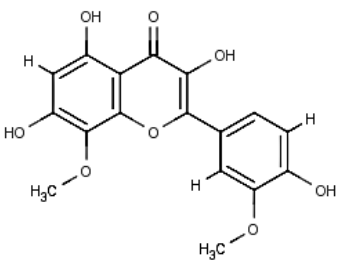

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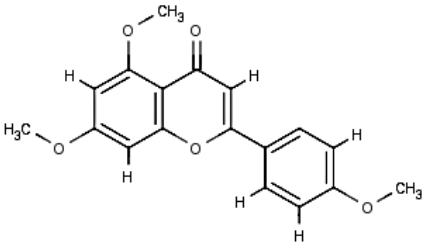

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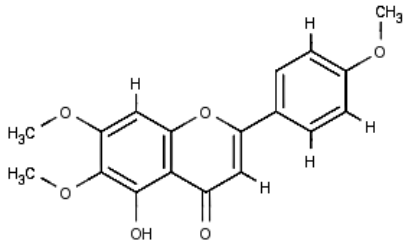

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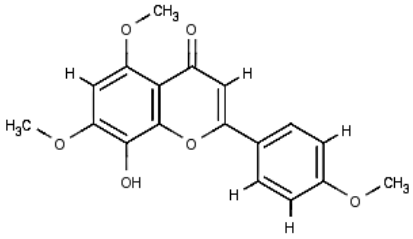

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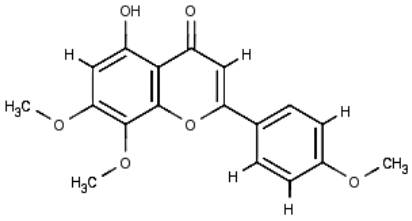

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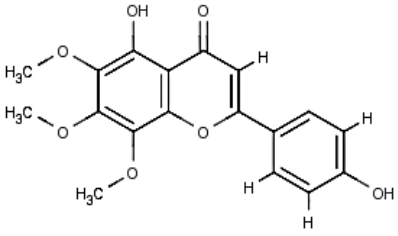

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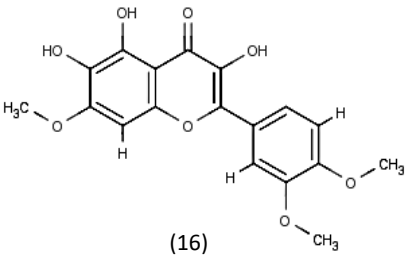

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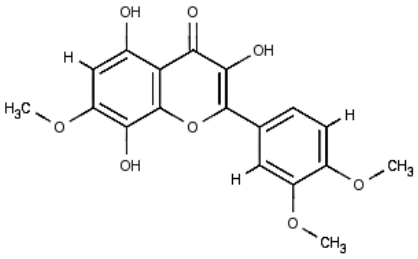

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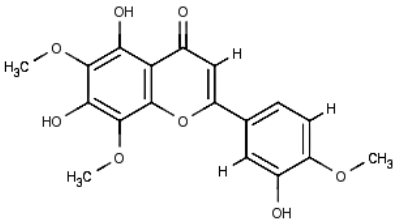

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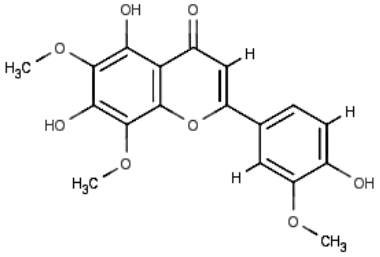

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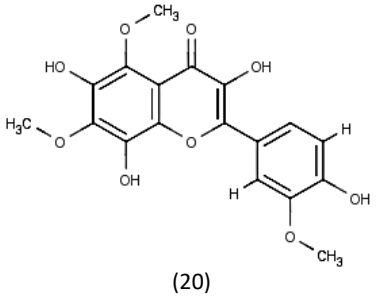

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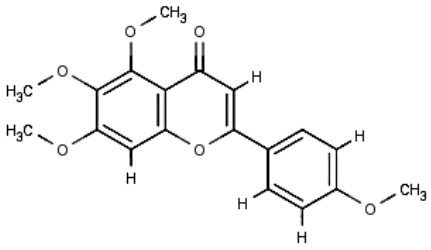

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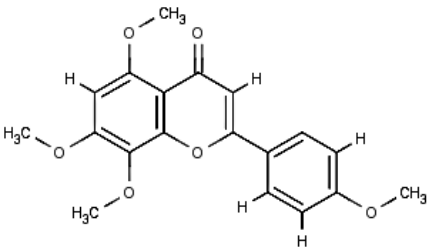

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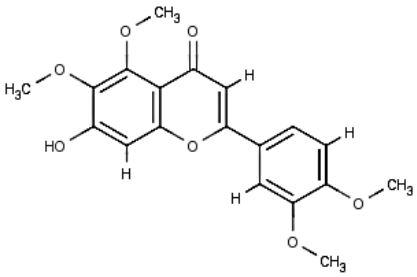

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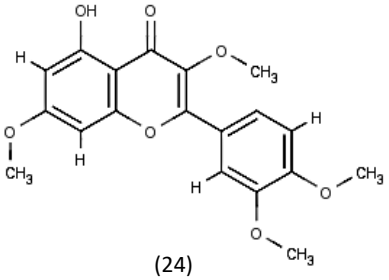

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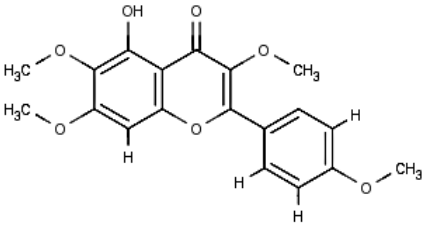

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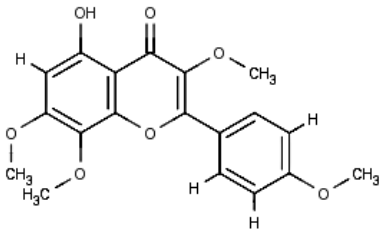

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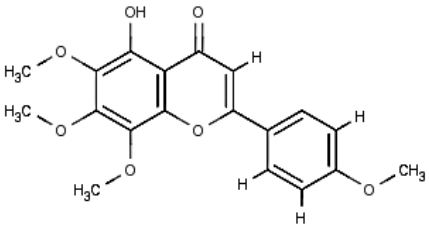

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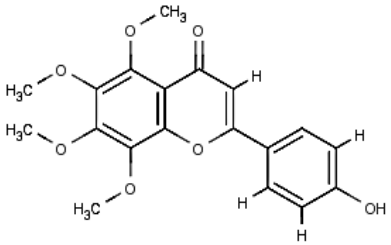

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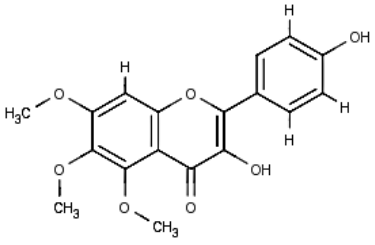

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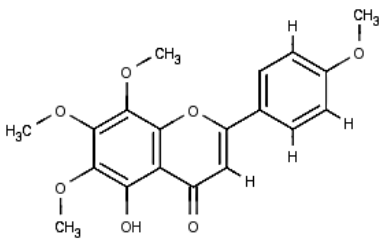

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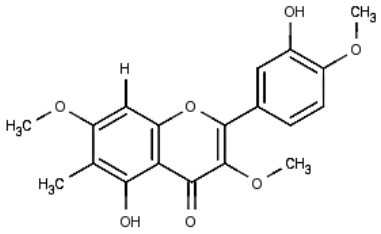

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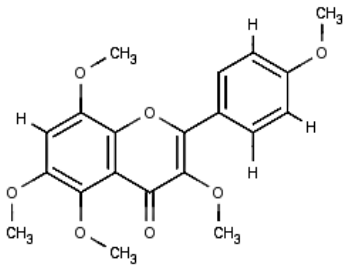

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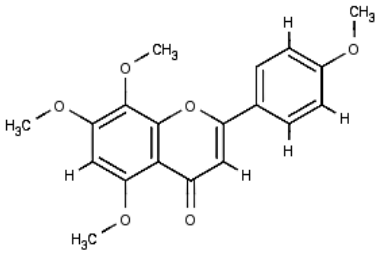

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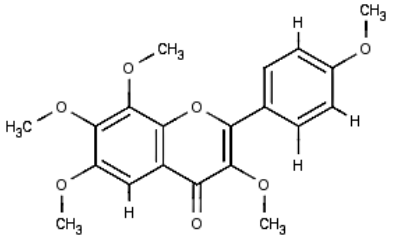

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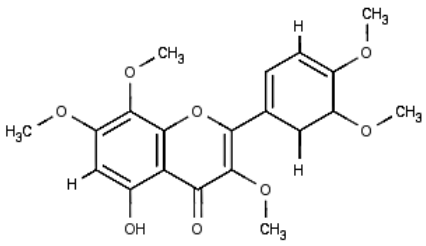

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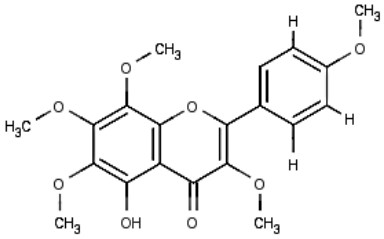

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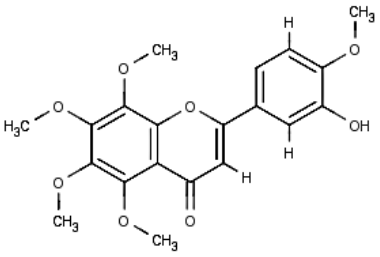

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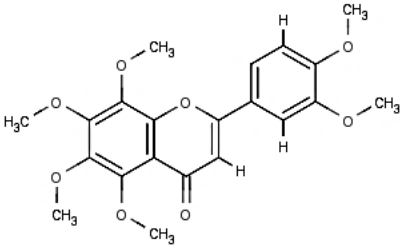

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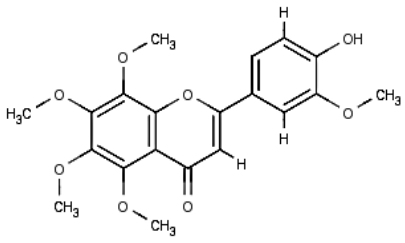

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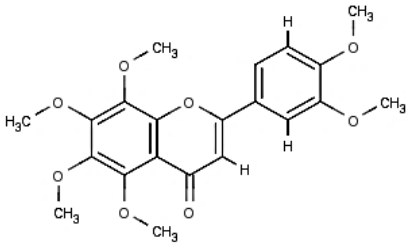

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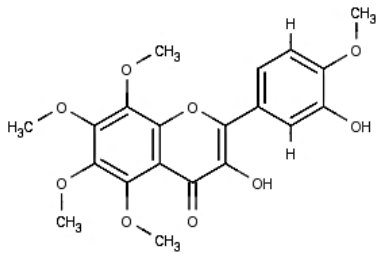

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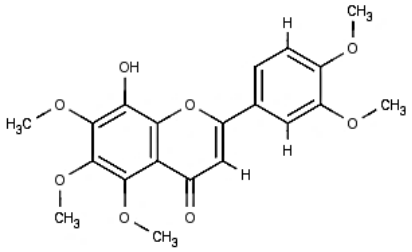

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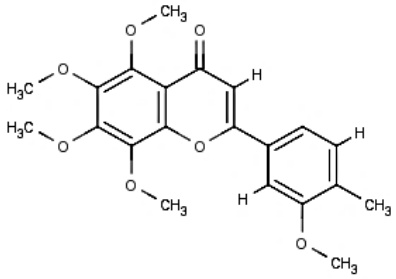

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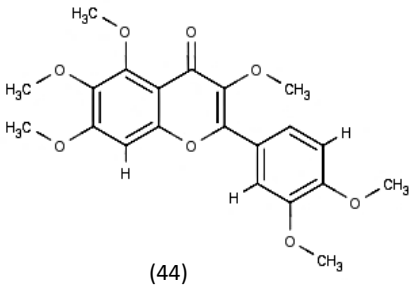

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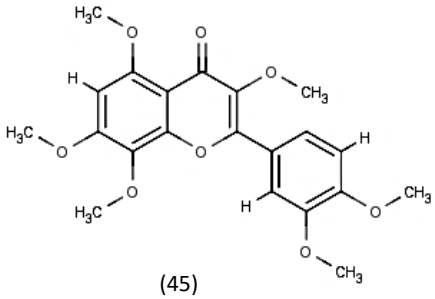

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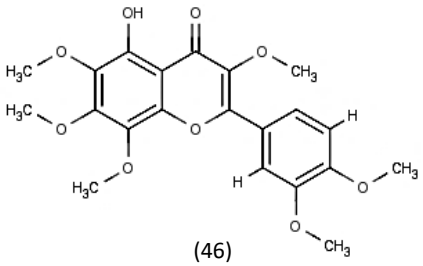

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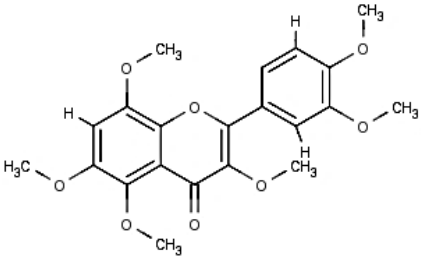

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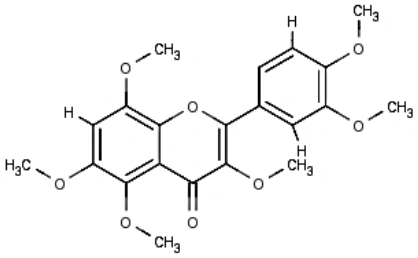

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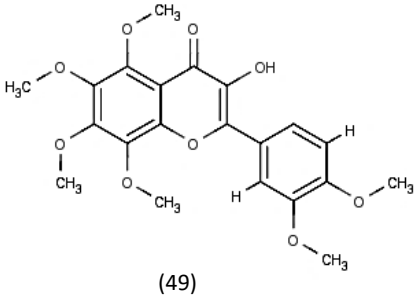

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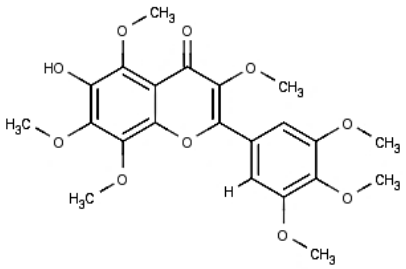

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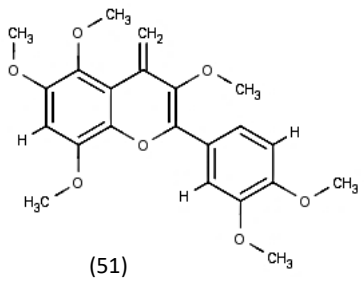

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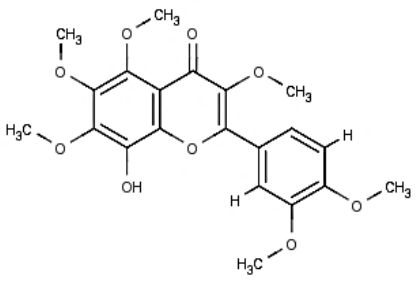

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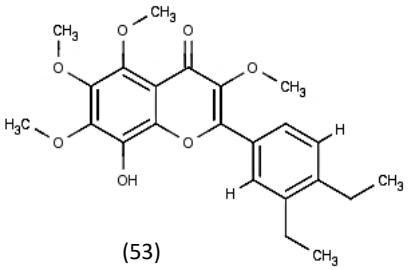

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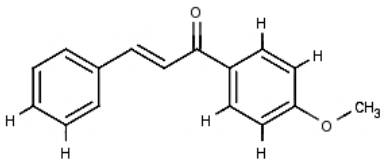

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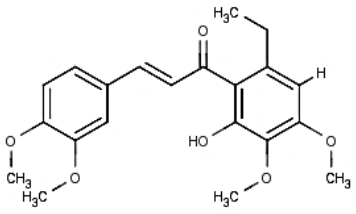

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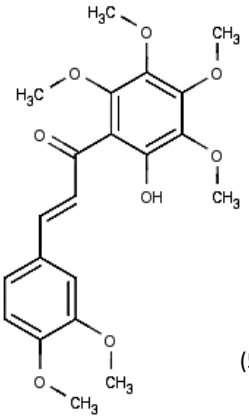

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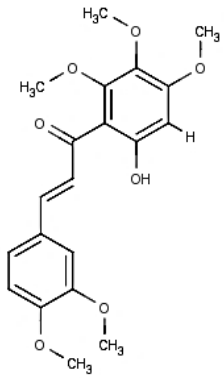

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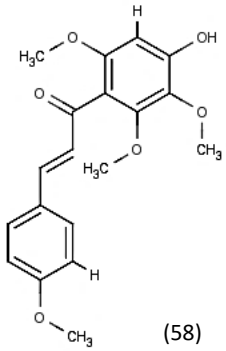

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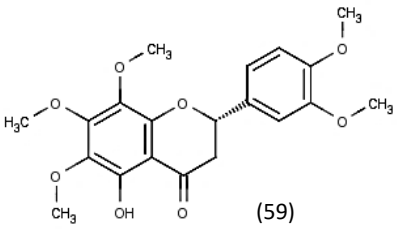

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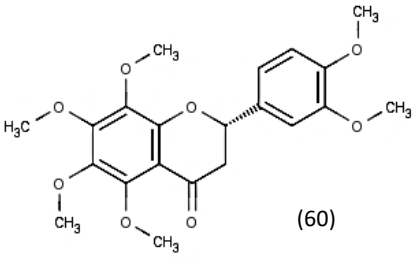

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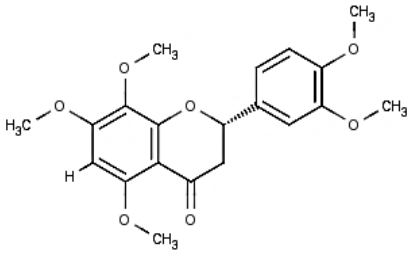

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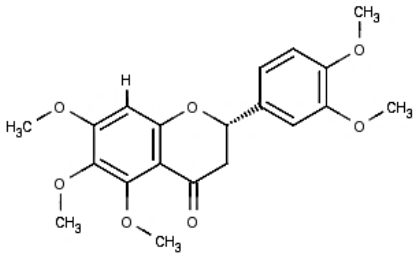

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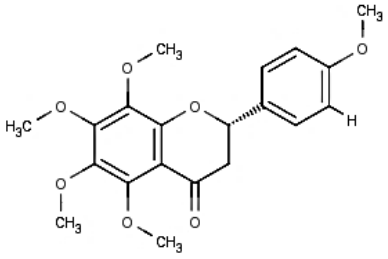

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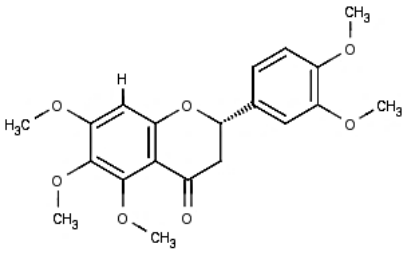

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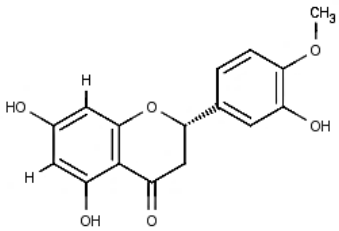

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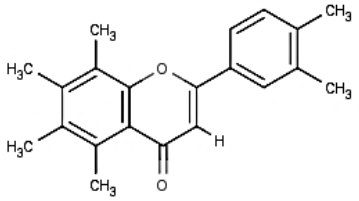

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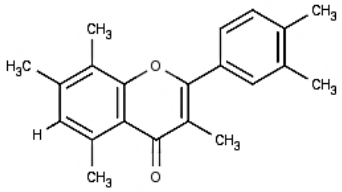

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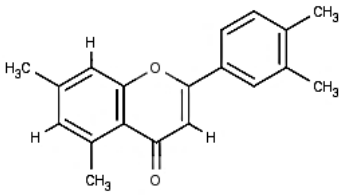

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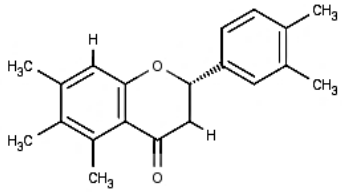


(69)

[Figure 4.1: **Structure of methylated flavonoids:**5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-chromen-4-one, 5,7,8-trihydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-7-methoxy-4H-

chromen-4-one, 2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-7-methoxy-4H-chromen-4-one, 8-hydroxy-7-methoxy-2-(2-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-2-(4-hydroxyphenyl)-7,8-dimethoxy-3-methyl-4H-chromen-4-one, 5-hydroxy-2-(4-hydroxyphenyl)-7,8-dimethoxy-3-methyl-4H-chromen-4-one, 3,5,6-trihydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5,6,7,8-tetrahydroxy-4H-chromen-4-one, 3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-8-methoxy-4H-chromen-4-one, 5,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-6,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 8-hydroxy-5,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-7,8-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-2-(4-hydroxyphenyl)-6,7,8-trimethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,6-trihydroxy-7-methoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,8-trihydroxy-7-methoxy-4H-chromen-4-one, 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-6,8-dimethoxy-4H-chromen-4-one, 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-6,8-dimethoxy-4H-chromen-4-one, 3,6,8-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-5,7-dimethoxy-4H-chromen-4-one, 5,6,7-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-7-hydroxy-5,6-dimethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-7-hydroxy-5,6-dimethoxy-4H-chromen-4-one, 3-hydroxy-2-(4-hydroxyphenyl)-5,6,7-trimethoxy-4H-chromen-4-one, 5-hydroxy-3,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-6,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(4-hydroxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one, 3-hydroxy-2-(4-hydroxyphenyl)-5,6,7-trimethoxy-4H-chromen-4-one, 5-hydroxy-6,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,7-dimethoxy-6-methyl-4H-chromen-4-one, 3,5,6,8-tetramethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 3,6,7,8-tetramethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5-hydroxy-3,7,8-trimethoxy-4H-chromen-4-one, 5-hydroxy-3,6,7,8-tetramethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3-hydroxy-4-methoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one hydrate, 2-(4-hydroxy-3-methoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one, 3-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-8-hydroxy-5,6,7-trimethoxy-4H-chromen-4-one, 5,6,7,8-tetramethoxy-2-(3-methoxy-4-methylphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,6,7-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5-hydroxy-3,6,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,6,8-tetramethoxy-4H-chromen-4-one, 2-(2,5-dimethoxyphenyl)-3,6,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3-hydroxy-5,6,7,8-tetramethoxy-4H-chromen-4-one, 6-hydroxy-3,5,7,8-tetramethoxy-2-(3,4,5-trimethoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,6,8-tetramethoxy-4-methylidene-4H-chromene, 2-(3,4-dimethoxyphenyl)-8-hydroxy-3,5,6,7-tetramethoxy-4H-chromen-4-one, 2-(3,4-diethylphenyl)-8-hydroxy-3,5,6,7-tetramethoxy-4H-chromen-4-one, (2E)-1-(4-methoxyphenyl)-3-phenylprop-2-en-1-one, 2E)-3-(3,4-dimethoxyphenyl)-1-(6-ethyl-2-hydroxy-3,4-dimethoxyphenyl)prop-2-en-1-one,(2E)-3-(3,4-dimethoxyphenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one, (2E)-3-(3,4-dimethoxyphenyl)-1-(6-hydroxy-2,3,4-trimethoxyphenyl)prop-2-en-1-one, (2E)-1-(4-hydroxy-2,3,6-trimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one, (2S)-2-(3,4-dimethoxyphenyl)-5-hydroxy-6,7,8-trimethoxy-3,4-dihydro-2H-1-benzopyran-4-one, (2S)-2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxy-3,4-dihydro-2H-1-benzopyran-4-one, (2S)-2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy-3,4-dihydro-2H-1-benzopyran-4-one, (2S)-2-(3,4-dimethoxyphenyl)-5,7,8-trimethoxy-3,4dihydro-2H-1-benzopyran-4-one, (2S)-5,6,7,8-tetramethoxy-2-(4-methoxyphenyl)-3,4-dihydro-2H-1-benzopyran-4-one, (2S)-2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy-3,4-dihydro-2H-1-benzopyran-4-one, (2S)-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,4-dihydro-2H-1-benzopyran-4-one, 2-(3,4-dimethylphenyl)-5,6,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-3,5,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-5,7-dimethyl-4H-chromen-4-one, (2S)-2-(3,4-dimethylphenyl)-5,6,7-trimethyl-3,4-dihydro-2H-1-benzopyran-4-one]

ADME includes BBB test, Caco-2 permeability test, HIA test, P-gp inhibition test, MDCK permeability and PPB test. BBB permeability test is done to predict the compounds ability to penetrate into the BBB by calculating the logD/logK value. Compounds showing BBB^+ are able to cross the BBB and those showing BBB^- are not able to do so. Out of 69 compounds taken as ligands, 12 are BBB^+ and rest are BBB^- (table 4.1). Caco-2 permeability test is done to validate the suitability of the compounds for oral administration and to predict human intestinal absorption rate and drug efflux (Breemen, *et al*, 2005). It measures the *in vivo* absorption of the drug across the gut wall. HIA test (Human intestinal absorption) provides the information about the compound's absorption into bloodstream (Wessel, *et al*, 1998). This is important as a drug to be suitable, oral

administration; it should be absorbed into the bloodstream. Percentage of HIA is defined as the percentage of drug administered orally that reaches the hepatic portal vein. From the table, we can conclude that more than 70% of each of the ligands reaches the hepatic portal vein and 5 out of 69 have 100% HIA. P-gp means P-glycoprotein is a transport protein molecule (Pillans, *et al*, 2014). When a drug is actively transported by P-gp then its bioavailability reduces, therefore its inhibition is important (Amin, *et al*, 2013). Out of 69, 24 are non-inhibitor but rest 45 are inhibitors of P-gp. So, most of the drugs have good bioavailability. MDCK (Madin- Darby canine kidney) cells are the types of cells that show low expression of transporter proteins and low metabolic activity (Irvine, *et al*, 1999). MDCK permeability is linked to P-gp. It predicts the extent to which the drugs will efflux. PPB (Plasma protein binding) indicates the period of time the drugs will remain in the blood. Drugs interact with the plasma or tissue protein and form complex. It indicates the distribution of drugs. Drugs dynamics is important as only the free drug molecules will interact with the receptors on the targeted proteins. Means more the percentage of binding, poor was the drug. 95% free means 5% is free to actively work against the target. Here none of the ligands shows 100% binding, means some part of the drugs will remain free for the targeted proteins.

Drug-likeness property includes CMC-like rule (comprehensive medicinal chemistry) and Rule of five. CMC-like rule defines the drug-like characters of the selected ligands or compounds as compared to the CMC database. It indicates a ligand as qualified or not qualified on the basis of a range. As per CMC database, the qualifying range of log P is -0.4 to 5.6, that for molecular weight is 160 to 480, that for molar refractivity is 40 to 130 and that for total number of atoms is 20-70. Out of 69 only one compound is CMC not qualified (table4.2). Rule of five was given by Chris Lipinski. This rule stated that if a compound violates two or more conditions out of these following conditions like >5 hydrogen bond donors, molecular mass >500, calculated log P value >5, Sum of donors and acceptors should be >10, then they are said to have poor absorption with poor permeability. In this case, all the 69 ligands are suitable and passed the rule of five (table4.2). Ames test was developed by Bruce Ames, used to examine whether the compound taken as ligand is able to cause mutations in the DNA of the test organism or not. A positive test indicates that the compound is mutagenic and may

act as a carcinogen (as most of the cancers are related to mutation). But there is no complete association between a compound being mutagenic and carcinogenic always. (False positive and false negative are known). Here out of 69, 3 are non-mutagen and rest are mutagens (table4.2). Carcino rat test signifies that a given compound is able to produce carcinogenic effect in rats or not (Wanibuchi**,** *et al*, 2004). Out of 69, only one is Carcino rat negative, rest others are positive (table4.2). hERG expands as human ether-a-go-go-related gene. It is involved in cardiac repolarization and encodes for the gene involved in inward rectifying voltage-gated potassium channels in the heart. Inhibition of hERG prolongs the QT interval of the cardiac cycle and this leads to the potential fatal ventricular tachyarrhythmia causing cell death of the test organism. Not all compounds that exhibit hERG inhibition will proceed to cardiotoxicity but still, its inhibition is a sensitive measure to cross checks the compounds. Out of 69, 34 exhibits low risk whereas rest show medium risk (table4.2).

Table4.1: ADME properties of the ligands and standard inhibitors

	STANDARDS		**ADME PROPERTY**			
Standards	**BBB**	**Caco-2**	**HIA**	**MDCK**	**Pgp(I)**	**PPB**
1	BBB+	12.4418	91.7252	7.62836	Non-inhibitor	95.59209
2	BBB-	22.5596	93.50045	0.0822323	Inhibitor	89.86518
3	BBB+	23.037	99.28634	61.0892	Non-inhibitor	100
4	BBB+	19.5023	98.34936	0.0443177	Inhibitor	100

	LIGANDS		**ADME PROPERTY**			
Ligands	**BBB**	**Caco-2**	**HIA**	**MDCK**	**Pgp(I)**	**PPB**
1	BBB-	7.02526	88.18826	23.8531	Non-inhibitor	90.16013
2	BBB-	9.32409	88.18001	27.7465	Non-inhibitor	90.72856
3	BBB-	4.93945	78.34267	21.9216	Non-inhibitor	85.3466
4	BBB-	10.8691	78.33157	27.782	Non-inhibitor	90.00934
5	BBB-	37.3659	95.99601	182.247	Non-inhibitor	86.77201
6	BBB-	15.6876	93.59491	42.2482	Inhibitor	89.12084
7	BBB-	5.3654	87.82133	83.9141	Non-inhibitor	87.15776
8	BBB-	5.08813	87.82152	7.10806	Non-inhibitor	81.90835
9	BBB-	6.3241	76.28319	2.76593	Inhibitor	86.90252
10	BBB-	9.63521	76.304	35.2941	Non-inhibitor	79.61837

11	BBB+	55.3919	97.92488	2.91282	Non-inhibitor	89.23074
12	BBB-	33.065	96.48635	1.02959	Inhibitor	87.41127
13	BBB-	37.5234	96.48551	8.29032	Non-inhibitor	87.267
14	BBB-	37.5249	96.48879	5.85056	Inhibitor	87.33959
15	BBB-	9.12274	93.45203	33.6279	Inhibitor	86.59456
16	BBB-	3.1597	86.80217	0.584809	Non-inhibitor	79.07711
17	BBB-	7.6456	86.80483	10.0304	Non-inhibitor	78.7669
18	BBB-	8.20745	86.799	1.1177	Inhibitor	83.82541
19	BBB-	6.65579	86.79903	31.0397	Non-inhibitor	83.98422
20	BBB-	5.79119	73.19857	0.347829	Non-inhibitor	76.5246
21	BBB-	53.769	98.44068	0.43586	Inhibitor	88.11521
22	BBB-	54.9919	98.44068	2.46246	Inhibitor	88.01683
23	BBB-	30.4875	96.80767	0.140155	Inhibitor	85.89788
24	BBB-	24.7609	96.80845	7.72373	Non-inhibitor	80.41033
25	BBB-	25.2665	96.80636	7.02672	Non-inhibitor	81.4828
26	BBB-	30.4903	96.80845	32.6907	Non-inhibitor	81.57822
27	BBB-	36.4848	96.80636	1.12492	Inhibitor	86.66454
28	BBB-	36.4931	96.80987	4.19207	Inhibitor	86.60702
29	BBB-	9.13705	93.45634	3.1826	Non-inhibitor	82.20566
30	BBB-	36.4848	96.80636	1.12492	Inhibitor	86.66454
31	BBB-	7.33774	93.79605	5.12038	Inhibitor	82.58061
32	BBB-	53.4985	98.88618	11.6373	Inhibitor	83.27144
33	BBB-	54.9919	98.44068	2.46246	Inhibitor	88.01683
34	BBB-	51.2863	98.88618	43.6237	Inhibitor	82.46623
35	BBB-	38.2247	96.79591	7.04255	Non-inhibitor	78.62973
36	BBB-	31.6501	96.79433	7.00188	Inhibitor	80.58084
37	BBB-	43.1616	96.79536	0.286843	Inhibitor	84.80384
38	BBB-	54.0214	99.07504	0.0669953	Inhibitor	84.85934
39	BBB-	41.2261	96.79537	0.10887	Inhibitor	84.57317
40	BBB-	54.0214	99.07504	0.0669953	Inhibitor	84.85934
41	BBB-	41.2216	96.79375	0.0863988	Inhibitor	84.77709
42	BBB-	41.2216	96.79375	0.0863988	Inhibitor	84.77709
43	BBB-	53.3736	98.84629	0.0913785	Inhibitor	87.55638
44	BBB-	47.3048	99.07504	0.117108	Inhibitor	78.33853
45	BBB-	52.7895	99.07504	0.965244	Inhibitor	77.96442
46	BBB-	38.3489	96.39352	0.34293	Inhibitor	76.81297
47	BBB-	54.2909	99.07504	0.506149	Inhibitor	79.99046
48	BBB-	53.6117	99.07504	35.8771	Non-inhibitor	79.28555
49	BBB-	41.0259	96.39538	0.0938047	Inhibitor	78.08129
50	BBB-	42.0076	95.6224	0.0571157	Inhibitor	71.83945
51	BBB-	54.2805	98.02333	0.571395	Inhibitor	86.17298
52	BBB-	37.0482	96.39311	0.181877	Inhibitor	76.27377
53	BBB-	41.0379	96.90329	4.80685	Inhibitor	89.78122
54	BBB+	55.1765	100	52.6243	Inhibitor	91.74429

55	BBB+	47.4485	96.43149	0.0851212	Inhibitor	90.31974
56	BBB-	42.4072	97.03145	0.0464288	Inhibitor	86.36673
57	BBB-	43.0811	96.9028	0.0571744	Inhibitor	87.17774
58	BBB-	44.461	96.49206	0.18686	Non-inhibitor	88.27599
59	BBB-	41.4489	96.35932	0.127776	Inhibitor	85.94318
60	BBB+	54.057	98.89852	0.0674751	Inhibitor	85.16708
61	BBB+	51.2529	98.86539	0.0694854	Non-inhibitor	86.8745
62	BBB+	55.2708	98.86539	0.218232	Inhibitor	86.41294
63	BBB+	53.628	98.86539	0.629079	Inhibitor	87.51192
64	BBB+	51.2529	98.86539	0.0694854	Non-inhibitor	86.8745
65	BBB-	7.00371	87.19291	24.4257	Non-inhibitor	96.79283
66	BBB+	56.6935	100	45.9875	Inhibitor	93.22826
67	BBB+	56.6128	100	40.5913	Inhibitor	94.9859
68	BBB+	56.0673	100	51.765	Inhibitor	95.09543
69	BBB+	56.4306	100	41.1392	Inhibitor	94.64093

Note: HIA, Human Intestinal Absorption, CR, Carcino Rat, PPB, Plasma Binding Protein, MDCK, Madin-Darby Canine Kidney cells, Pgp-I, Pgp inhibition and BBB, Blood-Brain Barrier penetration, BBB+, penetrable to Blood-Brain Barrier, BBB- not penetrable to Blood Brain Barrier). Standard- **[1]** N-(5-methyl-1H-pyrazol-3-yl)-2-phenylquinazolin-4-amine, **[2]** 4-[4-[[4-chloro-3-(trifluoro-methyl)phenyl]carbamoylamino]phenoxy]-N-methylpyridine-2-carboxamide, **[3]** 2-methyl-5-propan-2-ylcyclohexa-2,5-diene-1,4-dione, **[4]** 2-[[4-[carboxymethyl-(4-methoxyphenyl) sulfonylamino] naphthalen-1-yl]-(methoxyphenyl) -sulfonyl- amino].

Table4.2: Drug likeness and toxicity properties of the ligands and the standard inhibitors.

Standard	**Drug-likeness property**		**Toxicity property**		
	CMC like rule	**Rule of five**	**Ames test**	**Carcino rat**	**hERG Inhibition**
1	Q	Suitable	M	Negative	MR
2	Q	Suitable	M	Negative	MR
3	Q	Suitable	M	Positive	LR
4	NQ	Suitable	NM	Negative	LR

Ligand	**Drug-likeness property**		**Toxicity property**		
	CMC Like Rule	**Rule of Five**	**Ames test**	**Carcino Rat**	**hERG Inhibition**
1	Q	Suitable	M	Positive	MR
2	Q	Suitable	M	Positive	MR
3	Q	Suitable	M	Positive	MR
4	Q	Suitable	M	Positive	MR
5	Q	Suitable	M	Positive	MR
6	Q	Suitable	M	Positive	MR

7	Q	Suitable	M	Positive	MR
8	Q	Suitable	M	Positive	MR
9	Q	Suitable	M	Positive	LR
10	Q	Suitable	M	Positive	LR
11	Q	Suitable	M	Positive	LR
12	Q	Suitable	M	Positive	LR
13	Q	Suitable	M	Positive	LR
14	Q	Suitable	M	Positive	LR
15	Q	Suitable	M	Positive	LR
16	Q	Suitable	M	Positive	LR
17	Q	Suitable	M	Positive	LR
18	Q	Suitable	M	Positive	LR
19	Q	Suitable	M	Positive	LR
20	Q	Suitable	NM	Positive	LR
21	Q	Suitable	M	Positive	MR
22	Q	Suitable	M	Positive	MR
23	Q	Suitable	M	Positive	MR
24	Q	Suitable	M	Positive	MR
25	Q	Suitable	M	Positive	MR
26	Q	Suitable	M	Positive	MR
27	Q	Suitable	M	Positive	MR
28	Q	Suitable	M	Positive	MR
29	Q	Suitable	NM	Positive	MR
30	Q	Suitable	M	Positive	MR
31	Q	Suitable	M	Positive	LR
32	Q	Suitable	M	Positive	MR
33	Q	Suitable	M	Positive	MR
34	Q	Suitable	M	Positive	MR
35	Q	Suitable	M	Positive	LR
36	Q	Suitable	M	Positive	LR
37	Q	Suitable	M	Positive	LR
38	Q	Suitable	M	Positive	LR
39	Q	Suitable	M	Positive	LR
40	Q	Suitable	M	Positive	LR
41	Q	Suitable	M	Positive	LR
42	Q	Suitable	M	Positive	LR
43	Q	Suitable	M	Positive	LR
44	Q	Suitable	M	Positive	LR
45	Q	Suitable	M	Positive	LR
46	Q	Suitable	M	Positive	LR
47	Q	Suitable	M	Positive	LR
48	Q	Suitable	M	Positive	LR
49	Q	Suitable	M	Positive	LR
50	Q	Suitable	M	Positive	LR
51	Q	Suitable	M	Positive	MR
52	Q	Suitable	M	Positive	LR

53	Q	Suitable	M	Positive	LR
54	Q	Suitable	M	Positive	MR
55	Q	Suitable	NM	Positive	MR
56	Q	Suitable	M	Positive	MR
57	Q	Suitable	M	Positive	MR
58	Q	Suitable	M	Positive	MR
59	Q	Suitable	M	Positive	LR
60	Q	Suitable	M	Positive	LR
61	Q	Suitable	M	Positive	MR
62	Q	Suitable	M	Positive	MR
63	Q	Suitable	M	Positive	MR
64	Q	Suitable	M	Positive	LR
65	Q	Suitable	M	Positive	MR
66	NQ	Suitable	M	Positive	MR
67	Q	Suitable	M	Positive	MR
68	Q	Suitable	M	Positive	MR
69	Q	Suitable	M	Negative	MR

Note: CLR, CMC like rule, RF, Rule of five, AT, Ames test, hERG-I, human ether-a-go-go related gene, CR, Carcino rat, M, Mutagen, NM, Non-mutagen, Q, Qualified, NQ, Not qualified, N-(5-methyl-1H-pyrazol-3-yl)-2-phenylquinazolin-4-amine,4-[4-[[4-chloro-3-(trifluoromethyl)phenyl] carb amoylamino]phenoxy]-N-methylpyridine-2-carboxamide,2-methyl-5-propan-2-ylcyclohexa-2,5-diene-1,4-dione,2-[[4-[carboxymethyl-(4-methoxyphenyl)sulfonylamino]naphthalen-1-yl]-(methoxyphenyl)-sulfonyl- amino]

4.1.2 Ligand-Protein Binding analysis

The ligands 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-chromen-4-one, 5,7,8-trihydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-7-methoxy-4H-chromen-4-one, 2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-7-methoxy-4H-chromen-4-one, 8-hydroxy-7-methoxy-2-(2-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-2-(4-hydroxyphenyl)-7,8-dimethoxy-3-methyl-4H-chromen-4-one, 5-hydroxy-2-(4-hydroxyphenyl)-7,8-dimethoxy-3-methyl-4H-chromen-4-one, 3,5,6-trihydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5,6,7,8-tetrahydroxy-4H-chromen-4-one, 3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-8-methoxy-4H-chromen-4-one, 5,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-6,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 8-hydroxy-5,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-7,8-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-2-(4-

hydroxyphenyl)-6,7,8-trimethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,6-trihydroxy-7-methoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,8-trihydroxy-7-methoxy-4H-chromen-4-one, 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-6,8-dimethoxy-4H-chromen-4-one, 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-6,8-dimethoxy-4H-chromen-4-one, 3,6,8-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-5,7-dimethoxy-4H-chromen-4-one, 5,6,7-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-7-hydroxy-5,6-dimethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-7-hydroxy-5,6-dimethoxy-4H-chromen-4-one, 3-hydroxy-2-(4-hydroxyphenyl)-5,6,7-trimethoxy-4H-chromen-4-one, 5-hydroxy-3,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-6,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(4-hydroxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one, 3-hydroxy-2-(4-hydroxyphenyl)-5,6,7-trimethoxy-4H-chromen-4-one, 5-hydroxy-6,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,7-dimethoxy-6-methyl-4H-chromen-4-one, 3,5,6,8-tetramethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 5,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 3,6,7,8-tetramethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5-hydroxy-3,7,8-trimethoxy-4H-chromen-4-one, 5-hydroxy-3,6,7,8-tetramethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, 2-(3-hydroxy-4-methoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one hydrate, 2-(4-hydroxy-3-methoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one, 3-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-8-hydroxy-5,6,7-trimethoxy-4H-chromen-4-one, 5,6,7,8-tetramethoxy-2-(3-methoxy-4-methylphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,6,7-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-5-hydroxy-3,6,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,6,8-tetramethoxy-4H-chromen-4-one, 2-(2,5-dimethoxyphenyl)-3,6,7,8-tetramethoxy-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3-hydroxy-5,6,7,8-tetramethoxy-4H-chromen-4-one, 6-hydroxy-3,5,7,8-tetramethoxy-2-(3,4,5-trimethoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethoxyphenyl)-3,5,6,8-tetramethoxy-4-methylidene-4H-chromene, 2-(3,4-

dimethoxyphenyl)-8-hydroxy-3,5,6,7-tetramethoxy-4H-chromen-4-one, 2-(3,4-diethylphenyl)-8-hydroxy-3,5,6,7-tetramethoxy-4H-chromen-4-one, (2E)-1-(4-methoxyphenyl)-3-phenylprop-2-en-1-one, 2E)-3-(3,4-dimethoxyphenyl)-1-(6-ethyl-2-hydroxy-3,4-dimethoxyphenyl)prop-2-en-1-one, (2E)-3-(3,4-dimethoxyphenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one, (2E)-3-(3,4-dimethoxyphenyl)-1-(6-hydroxy-2,3,4-trimethoxyphenyl)prop-2-en-1-one, (2E)-1-(4-hydroxy-2,3,6-trimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one, (2S)-2-(3,4-dimethoxyphenyl)-5-hydroxy-6,7,8-trimethoxy-3,4-dihydro-2H-1-benzopyran-4-one, (2S)-2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxy-3,4-dihydro-2H-1-benzopyran-4-one, (2S)-2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy-3,4-dihydro-2H-1-benzopyran-4-one, (2S)-2-(3,4-dimethoxyphenyl)-5,7,8-trimethoxy-3,4dihydro-2H-1-benzopyran-4-one, (2S)-5,6,7,8-tetramethoxy-2-(4-methoxyphenyl)-3,4-dihydro-2H-1-benzopyran-4-one, (2S)-2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy-3,4-dihydro-2H-1-benzopyran-4-one, (2S)-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,4-dihydro-2H-1-benzopyran-4-one, 2-(3,4-dimethylphenyl)-5,6,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-3,5,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-5,7-dimethyl-4H-chromen-4-one, (2S)-2-(3,4-dimethylphenyl)-5,6,7-trimethyl-3,4-dihydro-2H-1-benzopyran-4-one were docked against the targeted proteins for the prediction of binding score. Table 4.3 shows the result of molecular docking studies in the form of binding energy score. The score reveals the highest and lowest binding score of different ligands with different target proteins. It also shows the binding score of the standard inhibitors of the respective proteins (table 4.3). More negative the binding energy score is, more affinity the ligand has for the respective protein. For 1PFU (methionyl-tRNA synthetase), two ligands (68 and 69) showed better binding energy (-10.4 and -10.3 Kcal/mol respectively) as compared to the standard inhibitor (-10.1Kcal/mol). For 4F0F (Roco-4 Kinase), 8 ligands show more negative binding energy (ranging from -7.1 to -8.6 Kcal/mol) as compared to the standard (-7 Kcal/mol). For 4I5P (Brain permeable polo-like Kinase 2), all the ligands show more negative binding energy (ranging from -7 to -11.4 Kcal/mol) as compared to the standard (-6.7 Kcal/mol) and for 5CGJ (Crystal structure of murine keap-1), 58 ligands show more negative binding energy (ranging from -4.5 to -7.9 Kcal/mol). Ligands 66, 67, 68 and 69 depicted higher affinities towards 1PFU, 4F0F and 4I5P as indicated by more negative binding energy values (-7 to -11.4 Kcal/mol)

Table4.3: Comparative table of binding affinity of standards and ligands with targeted proteins

Standard No.	NAME	1PFU	4F0F	4I5P	5CGJ
1	N-(5-methyl-1H-pyrazol-3-yl)-2-phenylquinazolin-4-amine	-10.1			
2	4-[4-[[4-chloro-3-(trifluoromethyl) phenyl]carbamoylamino]phenoxy]-N-methylpyridine-2-carboxamide		-7		
3	2-methyl-5-propan-2-ylcyclohexa-2,5-diene-1,4-dione			-6.7	
4	2-[[4-[carboxymethyl-(4-methoxyphenyl)sulfonylamino]naphthalen-1-yl]-(4-methoxyphenyl)-sulfonylamino]acetic acid				-4.3

MF No.	NAME	1PFU	4F0F	4I5P	5CGJ
1	5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-chromen-4-one	-9.4	-7.6	-8.8	-7.4
2	5,7,8-trihydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-9.2	-7.4	-8.9	-5.8
3	2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-7-methoxy-4H-chromen-4-one	-9	-6.8	-8.8	-6.3
4	2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-7-methoxy-4H-chromen-4-one	-9	-6.7	-8.7	-7
5	8-hydroxy-7-methoxy-2-(2-methoxyphenyl)-4H-chromen-4-one	-8.1	-7.1	-8.9	-6.9
6	5-hydroxy-2-(4-hydroxyphenyl)-7,8-dimethoxy-3-methyl-4H-chromen-4-one	-7	-6.2	-8	-7.9
7	5-hydroxy-2-(4-hydroxyphenyl)-7,8-dimethoxy-3-methyl-4H-chromen-4-one	-7.3	-6.5	-8.8	-6.4
8	3,5,6-trihydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-8.4	-6	-8.9	-7
9	2-(3,4-dimethoxyphenyl)-5,6,7,8-tetrahydroxy-4H-chromen-4-one	-7.6	-6.8	-8.6	-6
10	3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-8-methoxy-4H-chromen-4-one	-6.9	-6.6	-8.7	-7.5
11	5,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-7.7	-6.3	-8.8	-5.6
12	5-hydroxy-6,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-8.8	-6.8	-8.4	-5.4
13	8-hydroxy-5,7-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-7.9	-6.2	-8.8	-5.7
14	5-hydroxy-7,8-dimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-7.6	-7.1	-8.7	-6.1
15	5-hydroxy-2-(4-hydroxyphenyl)-6,7,8-trimethoxy-4H-chromen-4-one	-7.2	-6.8	-8.1	-5.3
16	2-(3,4-dimethoxyphenyl)-3,5,6-trihydroxy-7-methoxy-4H-chromen-4-one	-8.2	-5.9	-8.6	-6.8
17	2-(3,4-dimethoxyphenyl)-3,5,8-trihydroxy-7-methoxy-4H-chromen-4-one	-6.9	-5.9	-8.6	-6.6
18	5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-6,8-dimethoxy-4H-chromen-4-one	-7.8	-6.9	-8.5	-4.3
19	5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-6,8-dimethoxy-4H-chromen-4-one	-7.5	-6.5	-8.7	-5.4
20	3,6,8-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-5,7-dimethoxy-4H-chromen-4-one	-7.1	-5.8	-7.9	-5.7
21	5,6,7-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-9	-6.1	-8.1	-5.4
22	5,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-7.5	-6.4	-8.6	-5.4

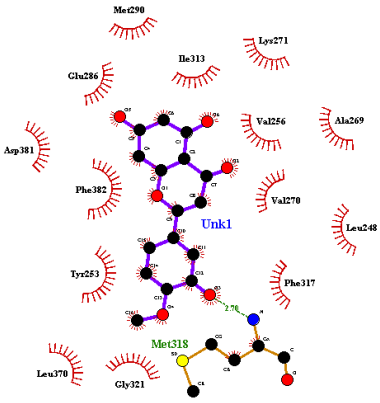
23	2-(3,4-dimethoxyphenyl)-7-hydroxy-5,6-dimethoxy-4H-chromen-4-one	-7.9	-6.6	-8.2	-4.2
24	2-(3,4-dimethoxyphenyl)-7-hydroxy-5,6-dimethoxy-4H-chromen-4-one	-7.9	-6.2	-7.7	-6.3
25	3-hydroxy-2-(4-hydroxyphenyl)-5,6,7-trimethoxy-4H-chromen-4-one	-6.8	-5.6	-7.4	-5
26	5-hydroxy-3,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-6.9	-6.5	-7.7	-6.2
27	5-hydroxy-6,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-7	-6.6	-7.6	-5.8
28	2-(4-hydroxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one	-7	-6.4	-7.6	-5.4
29	3-hydroxy-2-(4-hydroxyphenyl)-5,6,7-trimethoxy-4H-chromen-4-one	-7.3	-6.3	-8.2	-5.7
30	5-hydroxy-6,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-7.3	-6.5	-8.2	-5.1
31	5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,7-dimethoxy-6-methyl-4H-chromen-4-one	-7	-5.7	-7.5	-6.4
32	3,5,6,8-tetramethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-7	-6.3	-7	-4.7
33	5,7,8-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-7.5	-6.5	-7.3	-5.3
34	3,6,7,8-tetramethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-6.5	-6	-7.2	-4.8
35	2-(3,4-dimethoxyphenyl)-5-hydroxy-3,7,8-trimethoxy-4H-chromen-4-one	-6.8	-5.5	-7.7	-5.7
36	5-hydroxy-3,6,7,8-tetramethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	-6.6	-4.3	-7	-4.8
37	2-(3-hydroxy-4-methoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one	-7.4	-5.7	-7.4	-5.8
38	2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one hydrate	-7.3	-6.1	-7.5	-4.9
39	2-(4-hydroxy-3-methoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one	-7	-6.2	-7.9	-5.6
40	2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one	-7.3	-6.1	-7.3	-4.9
41	3-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-5,6,7,8-tetramethoxy-4H-chromen-4-one	-7	-6.3	-7.7	-5.8
42	2-(3,4-dimethoxyphenyl)-8-hydroxy-5,6,7-trimethoxy-4H-chromen-4-one	-7.1	-4.9	-7.3	-3.3
43	5,6,7,8-tetramethoxy-2-(3-methoxy-4-methylphenyl)-4H-chromen-4-one	-7.2	-6.4	-8.6	-5.6
44	2-(3,4-dimethoxyphenyl)-3,5,6,7-tetramethoxy-4H-chromen-4-one	-7	-6.1	-8	-3.6
45	2-(3,4-dimethoxyphenyl)-3,5,7,8-tetramethoxy-4H-chromen-4-one	-7	-4	-7.6	-4
46	2-(3,4-dimethoxyphenyl)-5-hydroxy-3,6,7,8-tetramethoxy-4H-chromen-4-one	-6.9	-4.9	-7.1	-4.1
47	2-(3,4-dimethoxyphenyl)-3,5,6,8-tetramethoxy-4H-chromen-4-one	-7.1	-5.7	-7.5	-4.1
48	2-(2,5-dimethoxyphenyl)-3,6,7,8-tetramethoxy-4H-chromen-4-one	-6.6	-5	-7.2	-5.7
49	2-(3,4-dimethoxyphenyl)-3-hydroxy-5,6,7,8-tetramethoxy-4H-chromen-4-one	-6.8	-6.2	-7.9	-5
50	6-hydroxy-3,5,7,8-tetramethoxy-2-(3,4,5-trimethoxyphenyl)-4H-chromen-4-one	-6.7	-4.5	-7.5	-5.7
51	2-(3,4-dimethoxyphenyl)-3,5,6,8-tetramethoxy-4-methylidene-4H-chromene	-7.2	-5.5	-7.8	-3.8

52	2-(3,4-dimethoxyphenyl)-8-hydroxy-3,5,6,7-tetramethoxy-4H-chromen-4-one	-7	-5.4	-7.8	-2.6
53	2-(3,4-diethylphenyl)-8-hydroxy-3,5,6,7-tetramethoxy-4H-chromen-4-one	-7.2	-5.7	-8.1	-6.3
54	(2E)-1-(4-methoxyphenyl)-3-phenylprop-2-en-1-one	-8.8	-6.2	-8.5	-6.3
55	2E)-3-(3,4-dimethoxyphenyl)-1-(6-ethyl-2-hydroxy-3,4-dimethoxyphenyl)prop-2-en-1-one	-7.3	-5.6	-8.1	-4.5
56	(2E)-3-(3,4-dimethoxyphenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one	-7.9	-5	-8	-4.5
57	(2E)-3-(3,4-dimethoxyphenyl)-1-(6-hydroxy-2,3,4-trimethoxyphenyl)prop-2-en-1-one	-7.9	-5.3	-7.2	-3.8
58	(2E)-1-(4-hydroxy-2,3,6-trimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one	-7.2	-5.4	-7.7	-5.7
59	(2S)-2-(3,4-dimethoxyphenyl)-5-hydroxy-6,7,8-trimethoxy-3,4-dihydro-2H-1-benzopyran-4-one	-7.4	-6.5	-8.1	-4.3
60	(2S)-2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxy-3,4-dihydro-2H-1-benzopyran-4-one	-7.6	-5.2	-7.2	-3.8
61	(2S)-2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy-3,4-dihydro-2H-1-benzopyran-4-one	-7.3	-6	-7.6	-5.1
62	(2S)-2-(3,4-dimethoxyphenyl)-5,7,8-trimethoxy-3,4dihydro-2H-1-benzopyran-4-one	-7.4	-6	-8.3	-4.8
63	(2S)-5,6,7,8-tetramethoxy-2-(4-methoxyphenyl)-3,4-dihydro-2H-1-benzopyran-4-one	-7.3	-6.3	-7.7	-4.8
64	(2S)-2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy-3,4-dihydro-2H-1-benzopyran-4-one	-7.4	-6	-7.8	-5.1
65	(2S)-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,4-dihydro-2H-1-benzopyran-4-one	-9.6	-6.9	-8.9	-6.2
66	2-(3,4-dimethylphenyl)-5,6,7,8-tetramethyl-4H-chromen-4-one	-9.6	-8.6	-11.3	-4.9
67	2-(3,4-dimethylphenyl)-3,5,7,8-tetramethyl-4H-chromen-4-one	-8.6	-8	-11.4	-4.9
68	2-(3,4-dimethylphenyl)-5,7-dimethyl-4H-chromen-4-one	-10.4	-7.4	-10.6	-5.2
69	(2S)-2-(3,4-dimethylphenyl)-5,6,7-trimethyl-3,4-dihydro-2H-1-benzopyran-4-one	-10.3	-8.2	-10.9	-5.2

Note: MF- methylated flavonoids

4.1.3 Ligplot analysis

Ligplot is the software that generates a 2D diagram showing ligand-protein interaction. It reveals the number of hydrogen bonds and hydrophobic interactions, as well as with which amino acids of the protein the ligand is interacting and the chain number also. Pattern of interaction between ligand and protein is shown in figure 4.2. Position and name of amino acids involved in the interaction pattern such as hydrogen bonding and hydrophobic interaction are given in table 4.4.



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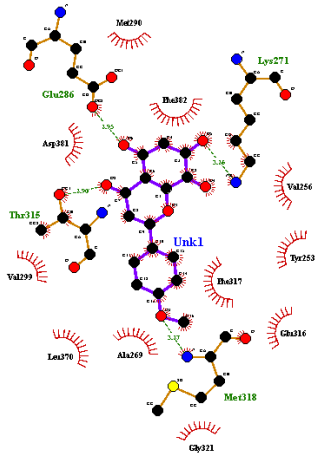

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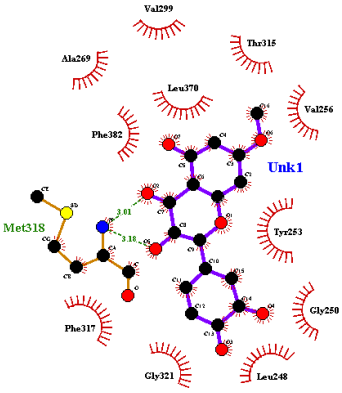

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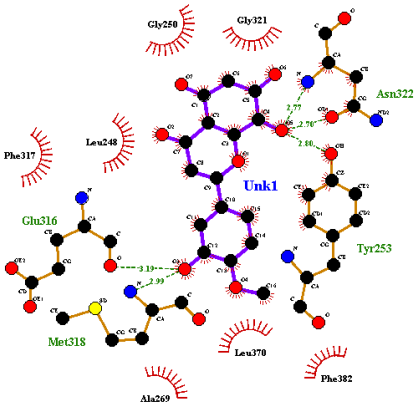

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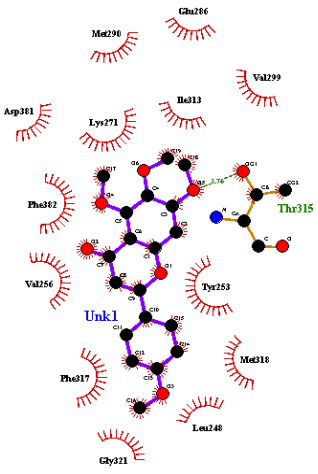

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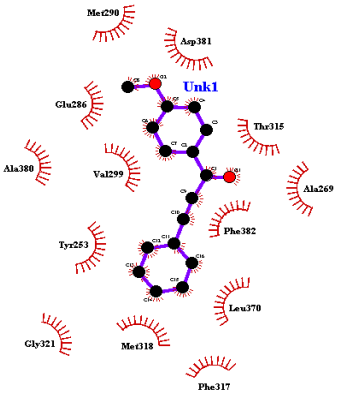

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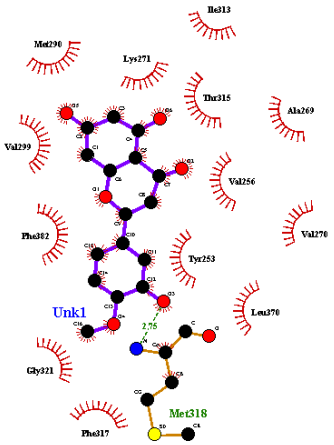
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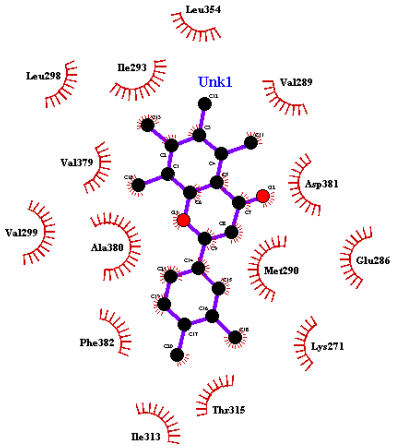
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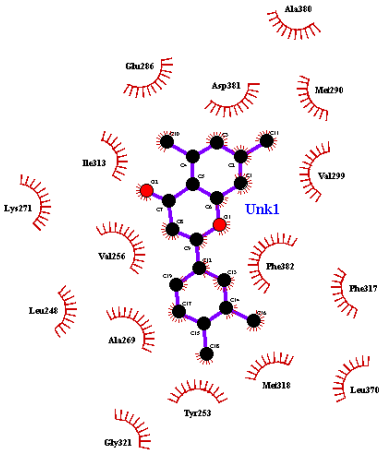
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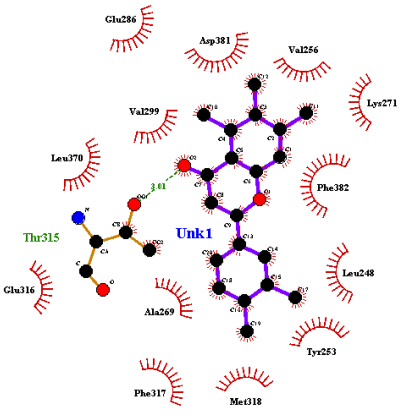
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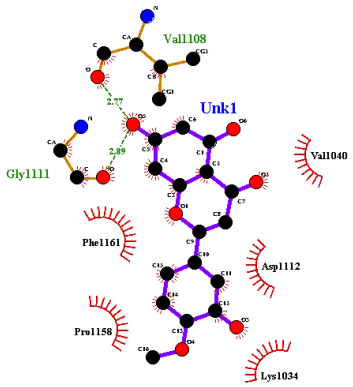

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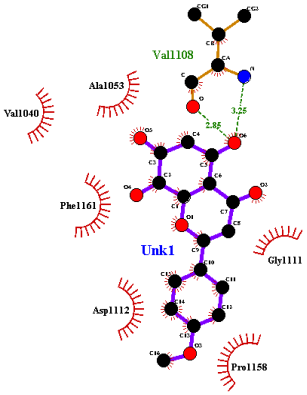

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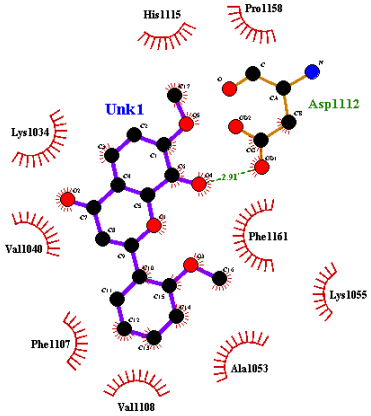

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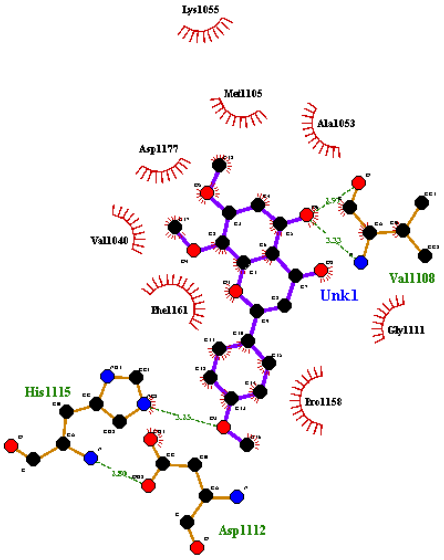

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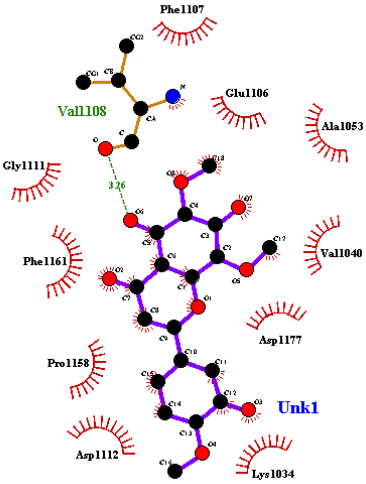

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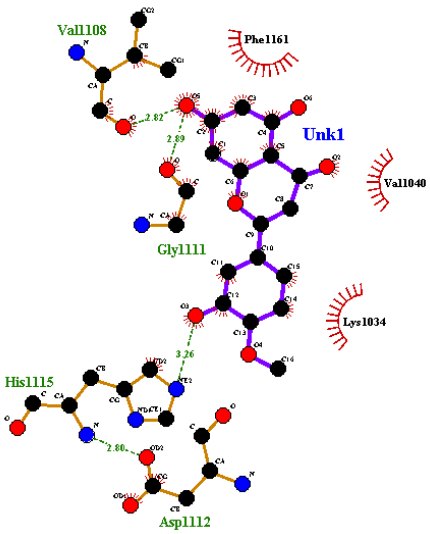

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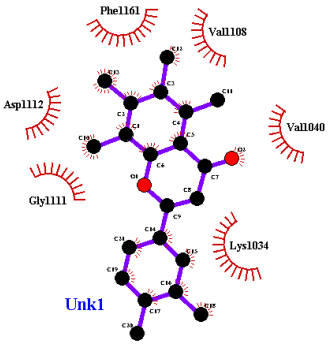
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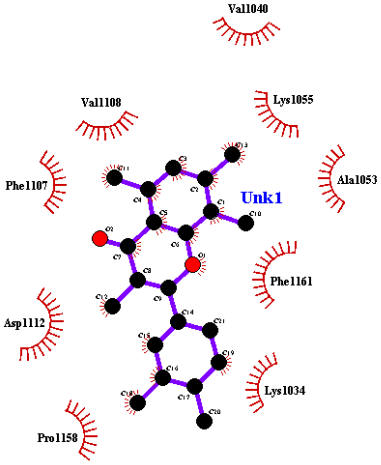
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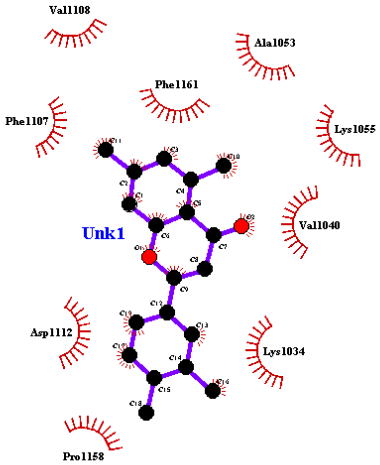
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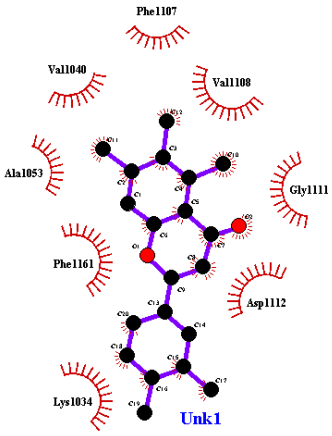
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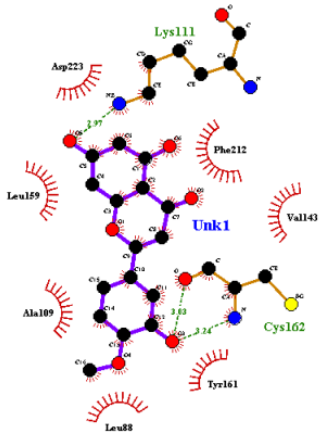

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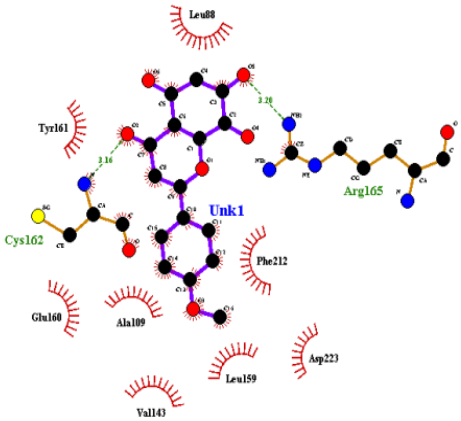

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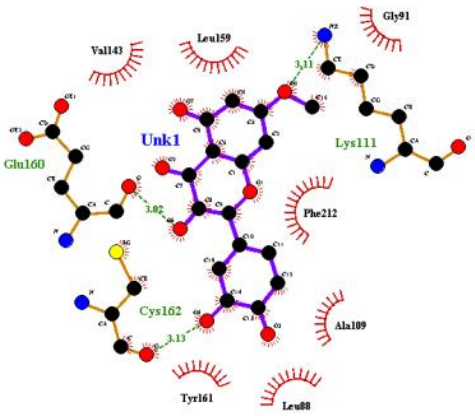

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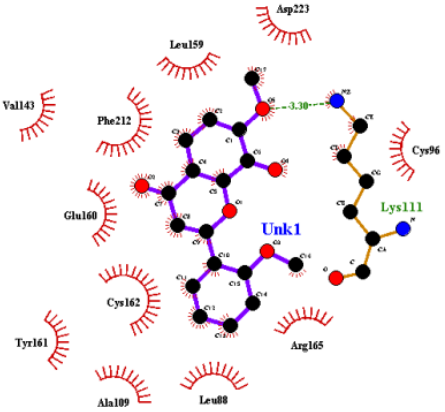

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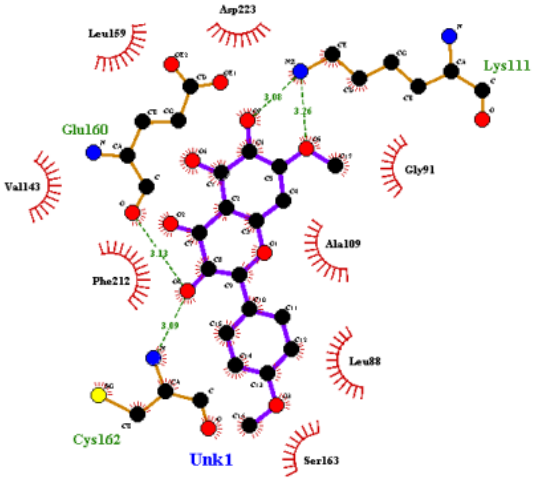

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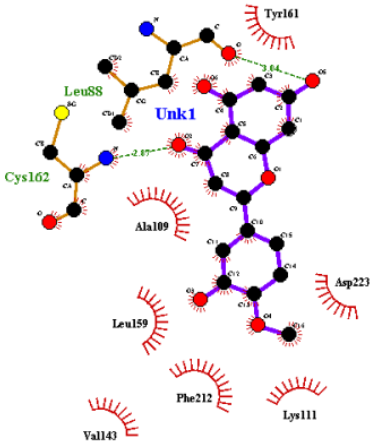

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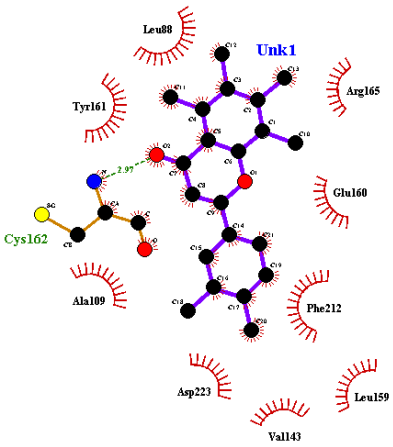
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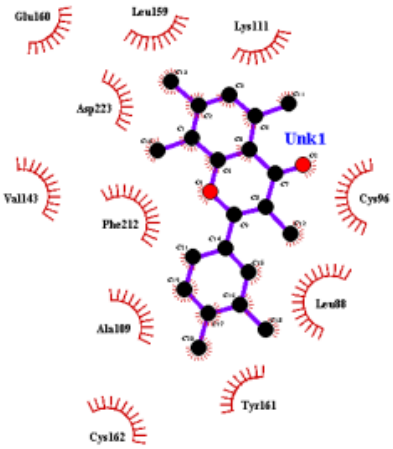
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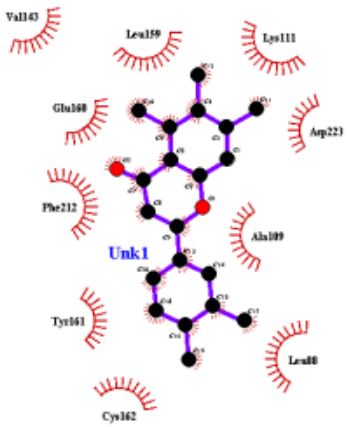
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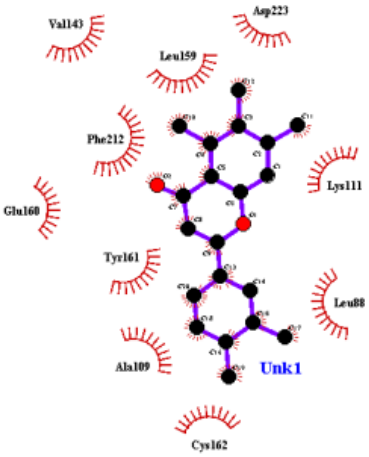
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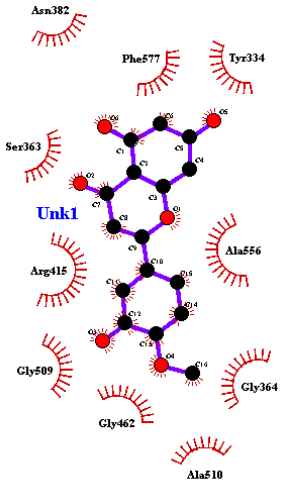

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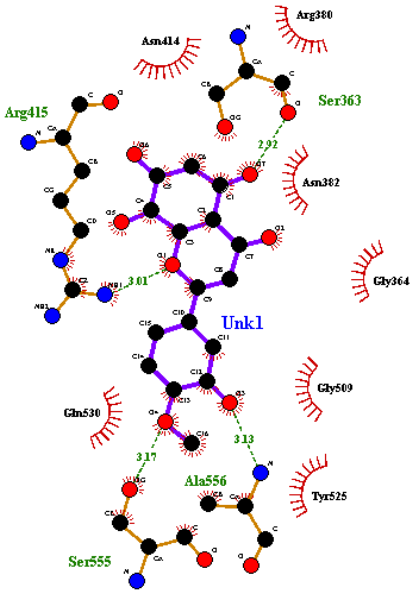

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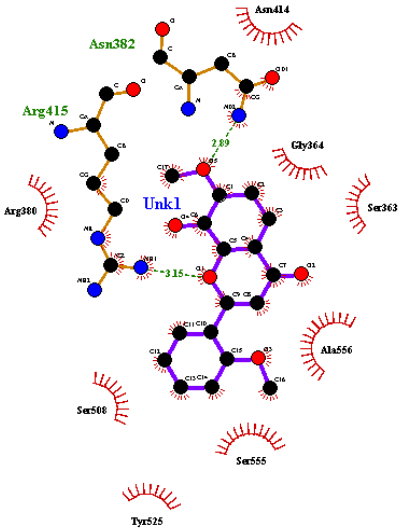

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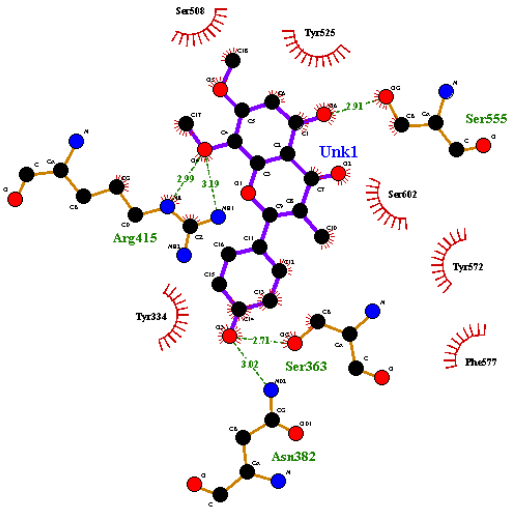

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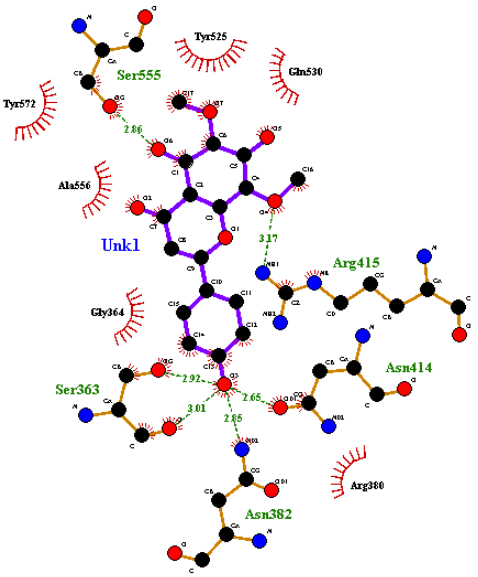

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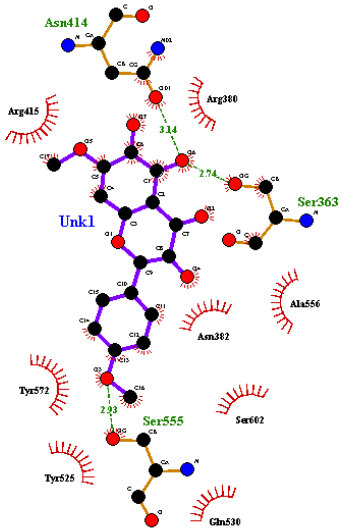

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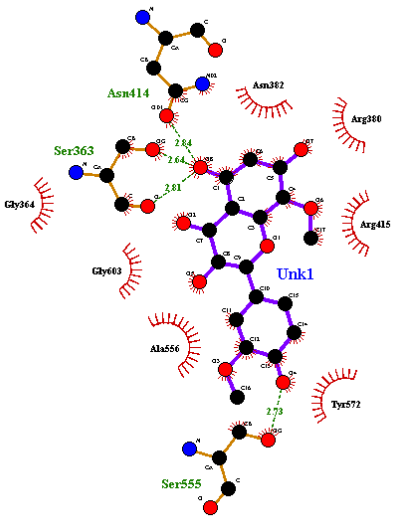

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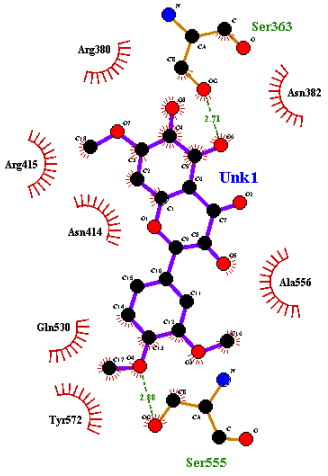

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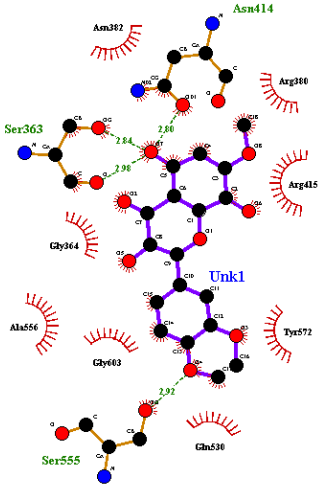

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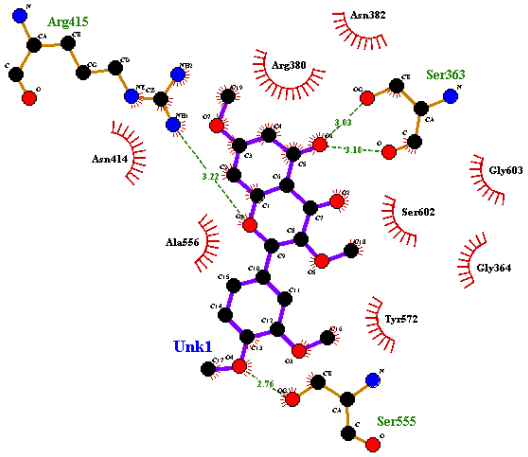


(40)

Figure 4.2: Ligplot showing amino acids of the target protein involved in interaction with ligand:(1)Methionyl tRNA synthetase and ligand 1, (2)Methionyl tRNA synthetase and ligand 2, (3)Methionyl tRNA synthetase and ligand 3, (4)Methionyl tRNA synthetase and ligand 4, (5)Methionyl tRNA synthetase and ligand 21, (6)Methionyl tRNA synthetase and ligand 54, (7)Methionyl tRNA synthetase and ligand 65, (8)Methionyl tRNA synthetase and ligand 66, (9)Methionyl tRNA synthetase and ligand 68, (10)Methionyl tRNA synthetase and ligand 69(11)Roco-4 Kinase and ligand 1, (12)Roco-4 Kinase and ligand 2, (13)Roco-4 Kinase and ligand 5, (14)Roco-4 Kinase and ligand 14, (15)Roco-4 Kinase and ligand 18, (16)Roco-4 Kinase and ligand 65, (17)Roco-4 Kinase and ligand 66, (18)Roco-4 Kinase and ligand 67, (19)Roco-4 Kinase and ligand 68, (20)Roco-4 Kinase and ligand 69, :(21)Brain permeable polo-like kinase and ligand 1, (22)Brain permeable polo-like kinase and ligand 2, (23)Brain permeable polo-like kinase and ligand 3, (24)Brain permeable polo-like kinase and ligand 5, (25)Brain permeable polo-like kinase and ligand 8, (26)Brain permeable polo-like kinase and ligand 65, (27)Brain permeable polo-like kinase and ligand 66, (28)Brain permeable polo-like kinase and ligand 67, (29)Brain permeable polo-like kinase and ligand 68, (30)Brain permeable polo-like kinase and ligand 69, (31)Murine Keap 1 and ligand 1, (32)Murine Keap 1 and ligand 4, (33)Murine Keap 1 and ligand 5, (34)Murine Keap 1 and ligand 6, (35)Murine Keap 1 and ligand 7, (36)Murine Keap 1 and ligand 8, (37)Murine

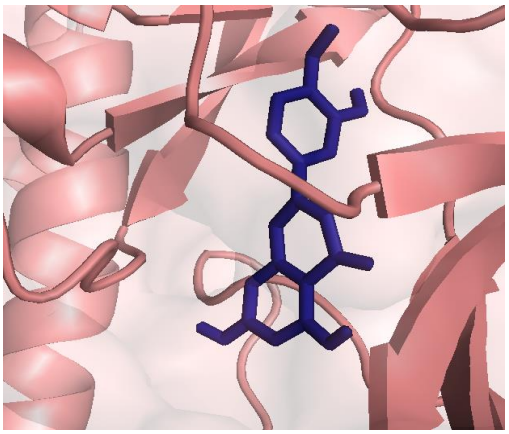
Keap 1 and ligand 10, (38)Murine Keap 1 and ligand 16, (39)Murine Keap 1 and ligand 17, (40)Murine Keap 1 and ligand 24.

Table 4.4: List of ligands exhibiting hydrogen bonds and hydrophobic interactions

Complex name	Residues involved in hydrophobic interactions	Hydrogen bonding residues
1PFU-ligand 1	Met 290, Lys 271, Ile 313, Glu 286, Val 256, Ala269, Asp 381, Phe 382, Val 270, Leu 248, Tyr 253, Phe 317, Leu 370, Gly 371	Met 318
1PFU-ligand 2	Met 290, Phe 382, Val 256, Tyr 253, Phe 317, Leu 370, Ala269, Arg 381, Val 229, Glu316, Gly 321	Glu 286, Lys 271, Met 318, Thr 315
1PFU-ligand 3	Val 299, Thr 315, Ala 269, Leu 370, Phe 382, Val 286, Tyr 253, Phe 317, Gly 250, Gly 321, Leu 248	Met 318
1PFU-ligand 4	Gly 250, Gly 321, Leu 248, Phe 317, Ala 269, Leu 270, Phe 382	Asn 322, Glu 316, Met 318, Tyr 253
1PFU-ligand 21	Met 290, Glu 286, Val 299, Asp 381, Lys 271, Ile 313, Phe 382, Val 256, Tyr 253, Phe 317, Met 318, Leu 248 Gly 321	Thr 315
1PFU-ligand 54	Met 290, Asp 381, Glu 286, Val 299, Ala 380, Thr 315, Tyr 253, Phe 382, Ala 269, Gly 321, Met 318, Leu 370, Phe 317	
1PFU-ligand 65	Met 290, Ile 313, Lys 271, Val 299, Phe 382, Thr 315, Ala 269, Val 256, Tyr 253, Val 270, Gly 321, Leu 370, Phe 317	Met 318
1PFU-ligand 66	Leu 354, Leu 298, Ile 293, Val 299, Val 289, Asp 381, Val 299, Ala 380, Met 290, Glu 286, Phe 382, Lys 271, Ile 313, Thr 315	
1PFU-ligand 68	Ala 380, Glu 286, Asp 381, Met 290, Ile 313, Val 299, Lys 271, Val 256, Phe 382, Leu 248, Ala 269, Phe 317, Gly 321, Tyr 253, Met 318, Leu 370	
1PFU-ligand 69	Glu 286, Asp 381, Val 256, Val 299, Lys 271, Leu 370, Phe 382, Glu 316, Leu 248, Ala 269, Tyr 253, Phe 317, Met 318	Thr 315
4F0F-ligand 1	Val 1040, Phe 1161, Asp 1112, Pro 1158, Lys 1034	Gly 1111
4F0F-ligand 2	Ala 1053, Val 1040, Phe 1161, Gly 1111, Asp 1112, Pro 1158	Val 1108
4F0F-ligand 5	His 1115, Pro 1158, Lys 1034, Val 1040, Phe 1161, Phe 1107, Lys 1055, Val 1108, Ala 1053	Asp 1112
4F0F-ligand 14	Lys 1055, Met 1105, Ala 1053, Asp 1177, Val 1040, Phe 1161, Gly 1111, Pro 1158	Val 1108, His 1115, Asp1112
4F0F-ligand 18	Phe 1107, Glu 1106, Ala 1053, Gly 1111, Phe 1161, Val 1040, Asp 1177, Pro 1158, Asp 1112, Lys 1034	Val 1108

4F0F-ligand 65	Phe 1161, Val 1040, Lys 1034	Val 1108, Gly1111, His 1115, Asp 1112
4F0F-ligand 66	Phe 1161, Val 1108, Asp 1112, Val 1040, Gly 1111, Lys 1034	
4F0F-ligand 67	Val 1040, Val 1108, Lys 1055, Phe 1107, Ala 1053, Phe 1161, Asp 1112, Lys 1034, Pro 1158	
4F0F-ligand 68	Val 1108, Phe 1161, Ala 1053, Lys 1055, Phe 1107, Lys 1055, Val 1040, Asp 1112, Lys 1034, Pro1158	
4F0F-ligand 69	Phe 1107, Val 1140, Val 1108, Ala 1053, Gly 1111, Phe 1161, Asp 1112, Lys 1034	
4I5P-ligand 1	Asp 223, Leu 159, Phe 212, Val 143, Ala 109, Tyr 161, Leu 88	Lys 111, Cys 162
4I5P-ligand 2	Leu 88, Tyr 161, Phe 212, Glu 160, Ala 109, Leu 159, Val 143, Asp 223	Cys 162, Arg 165
4I5P-ligand 3	Val 143, Leu 159, Gly 91, Phe 212, Ala 109, Tyr 161, Leu 88	Glu 160, Lys 111, Cys 162
4I5P-ligand 5	Asp 223, Leu 159, Val 143, Phe 212, Cys 162, Tyr 161, Arg 165, Ala 109, Leu 88	Lys 111
4I5P-ligand 8	Leu 159, Asp 223, Val 143, Gly 91, Phe 212, Ala 109, Leu 88, Ser 163	Glu 60, Lys 111, Cys 161
4I5P-ligand 65	Tyr 161, Ala 109, Asp 223, Leu 159, Phe 212, Lys 111, Val 143	Leu 88, Cys 162
4I5P-ligand 66	Leu 88, Arg 165, Tyr 161, Glu 160, Ala 109, Phe 212, Asp 223, Val 143, Leu 159	Cys 162
4I5P-ligand 67	Glu 160, Leu 159, Lys 111, Asp 223, Phe 212, Cys 96, Ala 189, Leu 88, Tyr 161, Cys 162	
4I5P-ligand 68	Val 143, Leu 159, Lys 111, Glu 168, Asp 223, Phe 212, Ala 109, Tyr 161, Leu 88, Cys 162	
4I5P-ligand 69	Val 143, Asp 223, Leu 159, Phe 212, Lys 111, Glu 160, Tyr 161, Leu 88, Ala 109, Cys 162	
5CGJ-ligand 1	Asn 382, Phe 577, Tyr 334, Ser 363, Ala 556, Arg 415, Gly 509, Gly 462, Gly 364	
5CGJ-ligand 4	Asn 414, Arg 380, Asn 382, Gly364, Gln 530, Gly 509, Tyr 525	Ala 556, Arg 415, Ser 555
5CGJ-ligand 5	Asn 414, Gly 364, Arg 380, Ser 363, Ser 508, Ala 556, Ser 555, Tyr 525	Arg 415, Asn382
5CGJ-ligand 6	Ser 508, Tyr 525, Ser 602, Tyr 334, Tyr 572, Tyr 334, Phe 577	Ser 363, Ser 555, Arg 415, Asn382
5CGJ- ligand 7	Tyr 525, Tyr 572, Gln 530, Ala 556, Gly 364, Arg 415	Asn 414, Ser 363, Ser 555, Arg415, Asn382

5CGJ-ligand 8	Arg 415, Arg 380, Ala 556, Asn 382, Tyr 572, Ser 602, Tyr 325, Glu 530	Asn 414, Ser 363, Ser 555
5CGJ-ligand 10	Asn 382, Arg 380, Gly 364, Arg 415, Gly 603, Ala 556, Tyr 572	Asn 414, Ser 363, Ser 555
5CGJ-ligand 16	Arg 380 , Asn 382, Arg 415, Asn 414, Ala 556, Gln 530, Tyr 572	Ser 363, Ser 555
5CGJ-ligand 17	Asn 382, Arg 380, Tyr 572, Gln 530, Gly 603, Ala 556, Gly 364	Asn 414, Ser 363, Ser 555
5CGJ-ligand 24	Asn 382, Arg 380, Asn 414, Gly 683 Ala 556, Ser 602, Gly 364, Tyr 572	Arg 415, Ser 363, Ser 555



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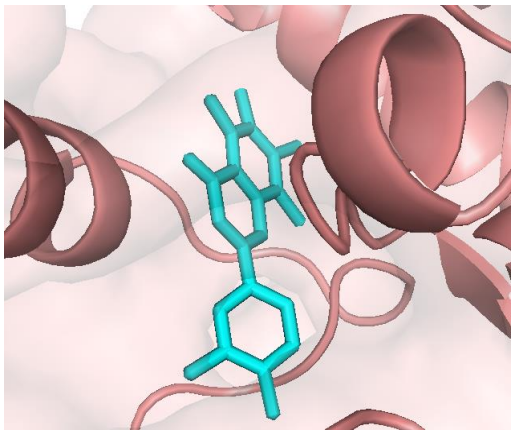

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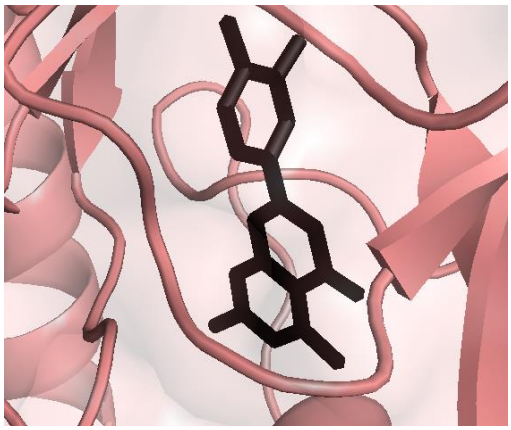

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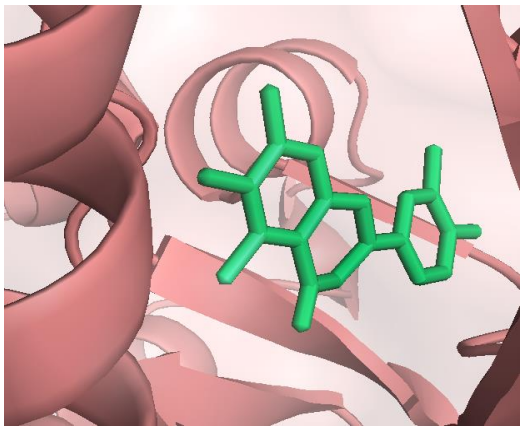

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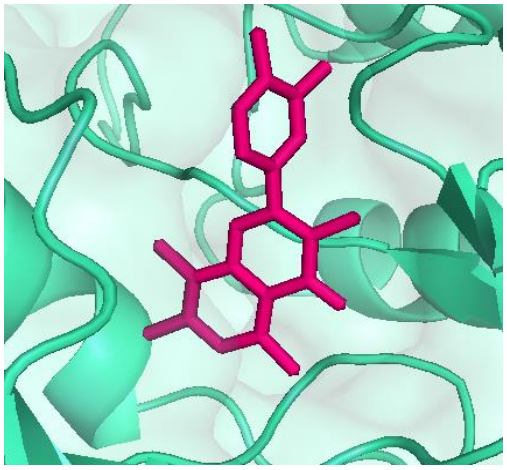

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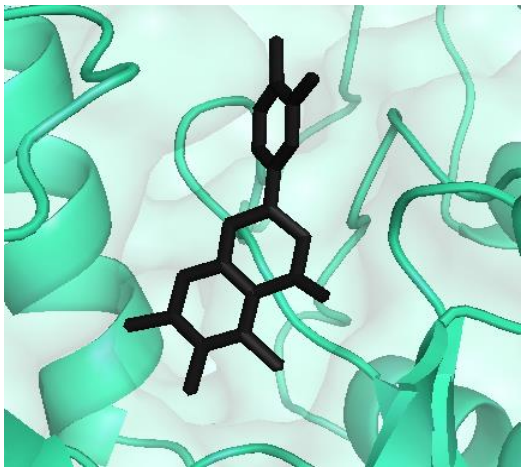

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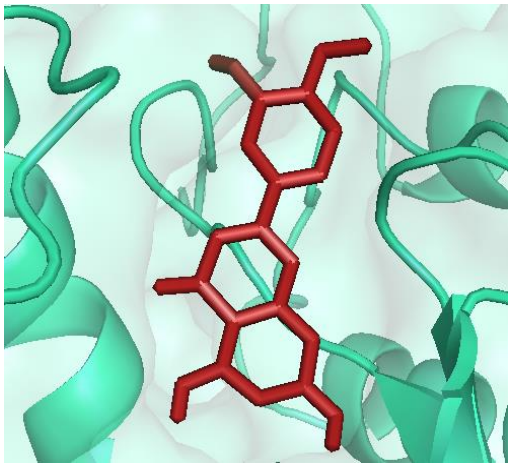

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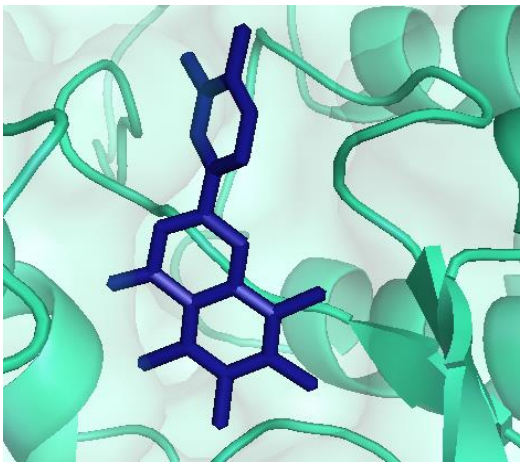

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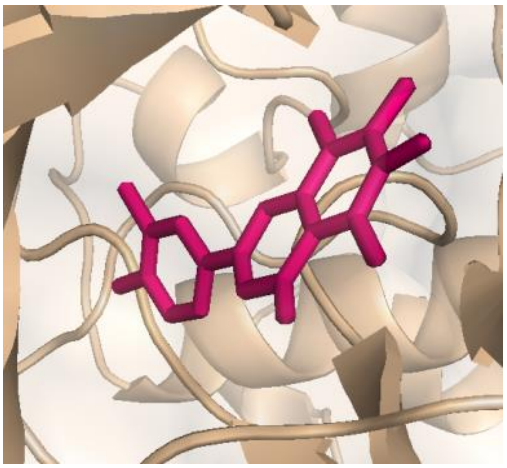

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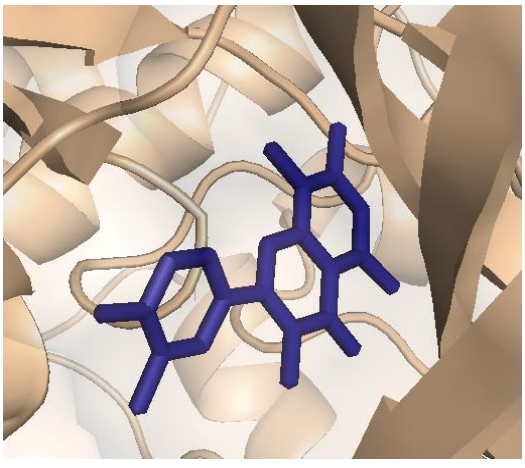

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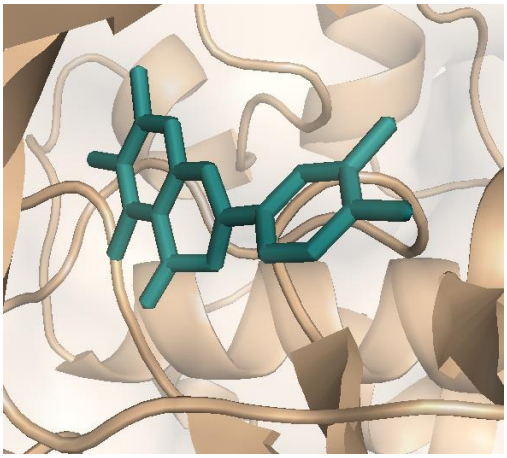

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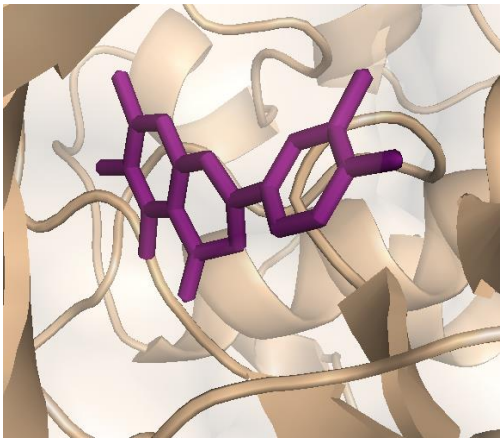

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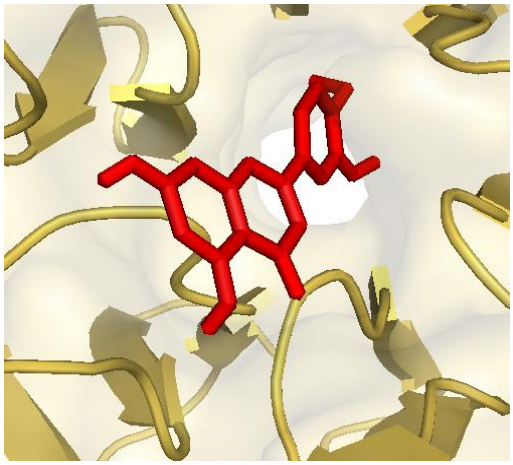

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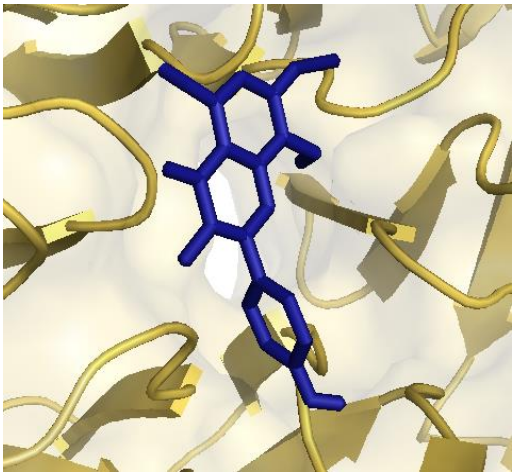

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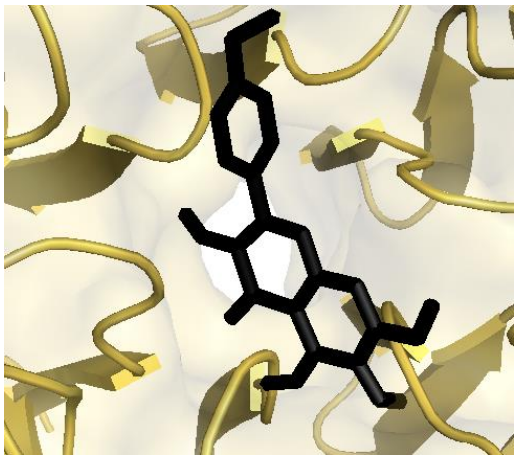

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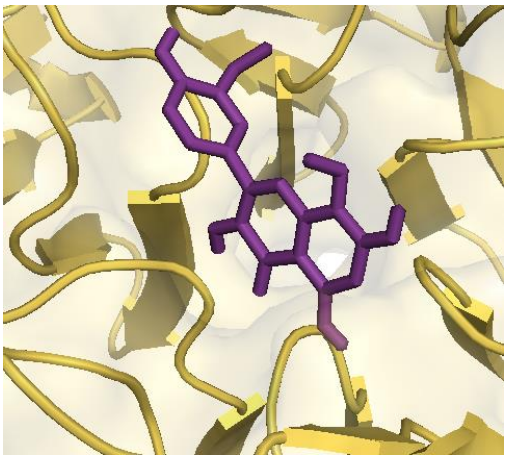


(16)

Figure 4.3: Surface structure of protein and ligand (best 4 of each protein)

(1)Methionyl tRNA synthetase and ligand 65, (2)Methionyl tRNA synthetase and ligand 66, (3)Methionyl tRNA synthetase and ligand 68, (4)Methionyl tRNA synthetase and ligand 69, (5)Roco-4 Kinase and ligand 1, (6)Roco-4 Kinase and ligand 66, (7)Roco-4 Kinase and ligand 67, (8)Roco-4 Kinase and ligand 69,(9)Brain permeable polo-like kinase and ligand 66, (10)Brain permeable polo-like kinase and ligand 67, (11)Brain permeable polo-like kinase and ligand 68, (12)Brain permeable polo-like kinase and ligand 69, (13)Murine Keap 1 and ligand 1, (14)Murine Keap 1 and ligand 6, (15)Murine Keap 1 and ligand 8, (16)Murine Keap 1 and ligand 10.

4.2 Results of laboratory techniques

4.2.1 Anticancer potential screening assay: MTT assay

To access the antiproliferative activity of compound 001, the colon cancer cell lines (HT29) were used and MTT assay was done. The results are shown in figure 4.4. The result showed that 001 exhibited moderate anticancer efficacy at test concentrations (0.5-0.04 mg/ml). About 55% growth inhibition activity was observed at higher test concentration (0.04 mg/ml) against colon cancer cell line.

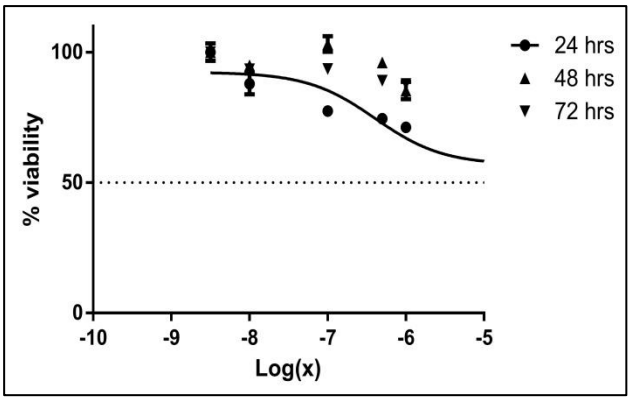


Figure 4.4: Viability of the cells decreases with increasing concentration of compound 001.

4.2.2 Antioxidant potential screening assay: DPPH radical scavenging activity.

Result showed that the antioxidant activity increased with increasing concentration of the sample (Figure 4.5). The radical scavenging activity of triphenylphosphineruthenium was found to be 75-80% at different test

concentration (1–20 μl/mL). It was found higher at higher concentrations (100μl/mL) (Mishra *et al.,* 2013)

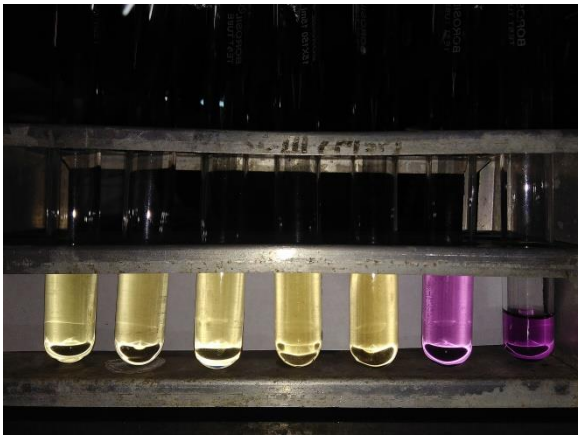
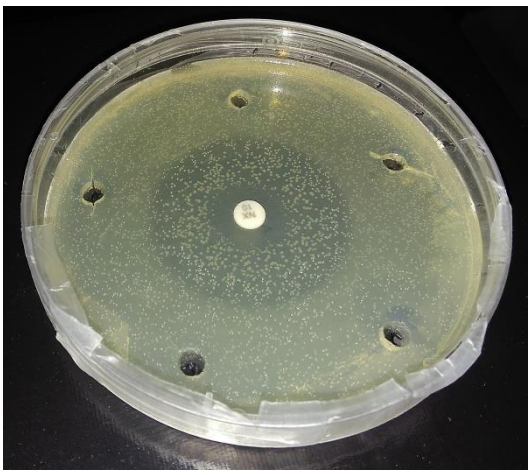


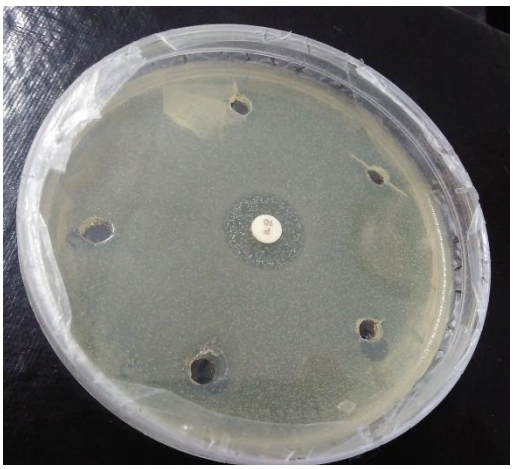
Figure 4.5: Percentage scavenging activity of compound 001

4.2.3 Antibacterial potential screening assay: Disc diffusion method

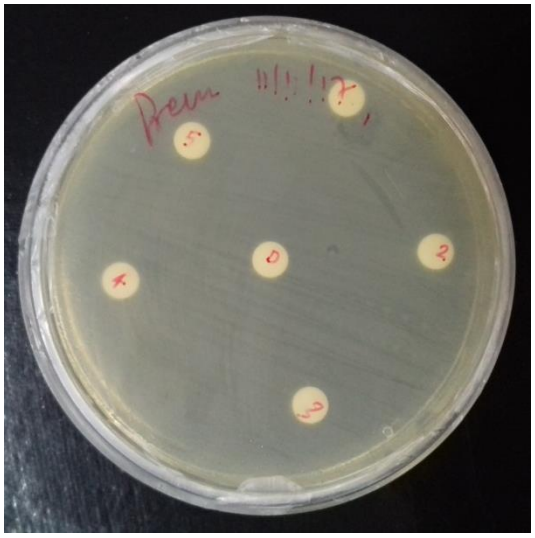
In this experiment norfloxacin and penicillin were used as standards. It was observed that no colonies emerged near the antibiotic norfloxacin, a few colonies grew after a rim of few millimetres and there was no effect of the antibiotic a few centimetres away it. In case of penicillin, inhibition was less as compared to norfloxacin. But in case of compound A, there was no significant inhibition of growing colonies.



(a)



(b)



(c)

Figure 4.6: Antibacterial activity by disc diffusion assay of (a) norfloxacin, (b) penicillin and (c) compound A.

Discussion

The different proteins involved in PD either for their upregulation or downregulation are found to be interconnected to each other. The imbalance in the ROS concentration leads to several pathophysiological conditions like diabetes, cancer and many neurodegenerative diseases like Parkinson's disease *(Barja, G., et al, 2004)* Alzheimer etc. So to protect its organelles, cells use different endogenous enzymatic and non-enzymatic pathways to inhibit ROS production by the production of antioxidant molecules like peroxidase, ubiquinone (ubiquitin proteasome complex) etc. Methionyl tRNA synthetase is an enzyme found in the cytoplasm that charge tRNA with the help of their cognate amino acids. Its function includes methionine ligase activity, tRNA binding, nucleotide binding, ATP binding, RNA binding etc. MARS 2, a gene in human codes for Methionyl tRNA synthetase (it is among those genes that are translocated inside the nucleus). Mutation in this gene leads to inhibition of complex I and increases the concentration of ROS *(Bayat, V., et al, 2012)*. There are no studies available regarding the inhibitors of Methionyl tRNA synthetase in humans. Till date, there is no identification of such compounds (whereas inhibitors of Methionyl tRNA synthetase of gram-positive bacteria, malarial parasites and other bacteria are known). In this study, we reported that methylated flavonoids 2-(3,4-dimethylphenyl)-5,7-dimethyl-4H-

chromen-4-one and (2S)-2-(3,4-dimethylphenyl)-5,6,7-trimethyl-3,4-dihydro-2H-1-benzopyran-4-one showed better potential against the protein Methionyl tRNA Synthetase as compared to the standards. They depicted 102.9% and 101.9% binding affinity respectively as compared to the standards.

Roco-4 Kinase is a nuclear protein which is 47% similar to LRRK2. It is different from it in the aspect of its isolation as a stable and soluble protein and it is used for the *in vivo* and *in vitro* study of the activity of LRRK2 in different diseased conditions like PD, as like LRRK2 it is also inhibited by LRRK2 inhibitors. Inhibitors of LRRK2 are known (they can also inhibit Roco-4 Kinase). By the help of kinase focused set screening, a potential novel drug has been discovered against Parkinson's i.e. 4-ethoxy-7H-pyrrolo[2,3-d]pyrimidin-2-amine *(Ding, X., et al, 2018).* It was able to penetrate into CNS and *in vivo* studies revealed that significant level of inhibition of Ser 935 phosphorylation is achieved by its administration in rat and mouse models. In this study we reported that methylated flavonoids 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-5,6,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-3,5,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-5,7-dimethyl-4H-chromen-4-one and (2S)-2-(3,4-dimethylphenyl)-5,6,7-trimethyl-3,4-dihydro-2H-1-benzopyran-4-one showed better potential against the protein Roco-4 Kinase as compared to the standards. They depicted 108.57%, 122.85%, 114.28%, 105.71% and 117.14% binding affinity respectively as compared to the standards.

Polo-like Kinase 2(PLK) is a protein found in the nucleus and belongs to serine/threonine kinase family having an N-terminal catalytic domain. Out of 5 discovered PLKs, PLK 2 and PLK 3 directly phosphorylate α-synuclein at Ser 129. The overexpression of PLK2 and PLK3 regulates α-synuclein phosphorylation at Ser 129*(Mbefo et al, 2010).* Phosphorylation of α-synuclein at Ser 129 results in the accumulation of α-synuclein filaments, then fibrils and then ultimately inclusions or Lewy bodies. This raises the chances of mutation in the kinase domain of LRRK2 which again increases the phosphorylation of α-synuclein *(Hayashita-Kinoh, H., et al, 2006).* In studies, it has been proved that LRRK2 proteins are found in the periphery of 10-80% of Lewy bodies. *(Anderson et al, 2006).* Inhibitors of PLK 2 are known**.** Recent studies have been done on PLK 2

inhibitors based on the tetrahydropteridin chemical scaffold and according to this study two compounds numbered C2 and C21 could be promising PLK 2 inhibitors *(Zhan, M.M., et al, 2018)*. Another study revealed that PLK 2 inhibition is a strong CNS pharmacological target that does not cause genotoxicity (chromosomal damage) and at doses and exposures to ELN582646 can inhibit the same by degradation of the particular protein *(Fitzerald, K., et al, 2013).* In this study we reported that methylated flavonoids 2-(3,4-dimethylphenyl)-5,6,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-3,5,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-5,7-dimethyl-4H-chromen-4-one and 2S)-2-(3,4-dimethylphenyl)-5,6,7-trimethyl-3,4-dihydro-2H-1-benzopyran-4-one showed better potential against the protein Roco-4 Kinase as compared to the standards. They depicted 168.65%, 170.14%, 158.2% and 162.68% binding affinity respectively as compared to the standards.

In Erk- Nrf2 pathway, the activation of Erk is required for the activation of Nrf2. The role of Nrf2 in Erk- Nrf2 pathway is the transcription of different genes involved in the production of antioxidant molecules. Nrf2 remains bound to Keap1 (in the cytoplasm). It is required for the transport of Nrf2 factor from outside the cell into the cytoplasm. In oxidative stress condition Keap1 gets modified, detaches itself from Nrf2 and then Nrf2 is released, stabilized by DJ-1 and enters into the nucleus by the help of ARE enhancers and transcription of above mentioned genes takes place. Inhibitors are known. Keap 1 inhibition can be in two different stages, first while it is outside the cell and second when it reaches the cytoplasm. Outside the cell Nrf2 requires Keap1 for its transport into the cytoplasm (as an anchor protein under stress condition). 1,4-Naphthoquinone (NQ) treatment inhibited mitogen-induced proliferation of lymphocytes via glutathionylation of Keap1. It increases total protein S-thionylation. Molecular docking studies revealed that NQ can disrupt Keap1/ Nrf2 interaction as it directly binds to the binding site of Nrf2 on Keap1 protein and inhibits the entry of Nrf2 into the cytoplasm *(Gambhir, L., et al, 2014).* But if inhibition occurs after the transport of Nrf2 inside the cytoplasm then only Nrf2 was able to enter the nucleus. Therefore Keap 1 is meant to be degraded. miR200, a regulatory mRNA controls the expression of Keap1 gene. It inhibits Keap1 3'-Untranslated region and results in its degradation *(Eades, G., et al, 2011).* In this study we reported that methylated flavonoids 5,7-dihydroxy-2-(3-

hydroxy-4-methoxyphenyl)-4H-chromen-4-one, 5-hydroxy-2-(4-hydroxyphenyl)-7,8-dimethoxy-3-methyl-4H-chromen-4-one, 3,5,6-trihydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one and 3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-8-methoxy-4H-chromen-4-one showed better potential against the protein Keap 1 as compared to the standards. They depicted 172.09%, 183.72%, 162.79% and 174.41% binding affinity respectively as compared to the standards.

Overall the inhibition of Methionyl tRNA synthetase gene i.e. MARS2 leads to the production of ROS. For the suppression of ROS, Nrf2 should transcribe the anti-oxidant genes which are again inhibited by the attachment of Keap1 to Nrf2 factor. Keap1 is associated with p62 (autophagic receptor) is responsible for the LRRK2 degradation as well as its analog Roco-4 Kinase. LRRK2 is also mutated by the Lewy bodies which are formed due to the aggregation of the α-synuclein protein. Phosphorylation of α-synuclein is due to the overexpression of PLK 2 and 3. All the proteins associated with Parkinson's disease are interrelated either by stimulating or inhibiting each other.

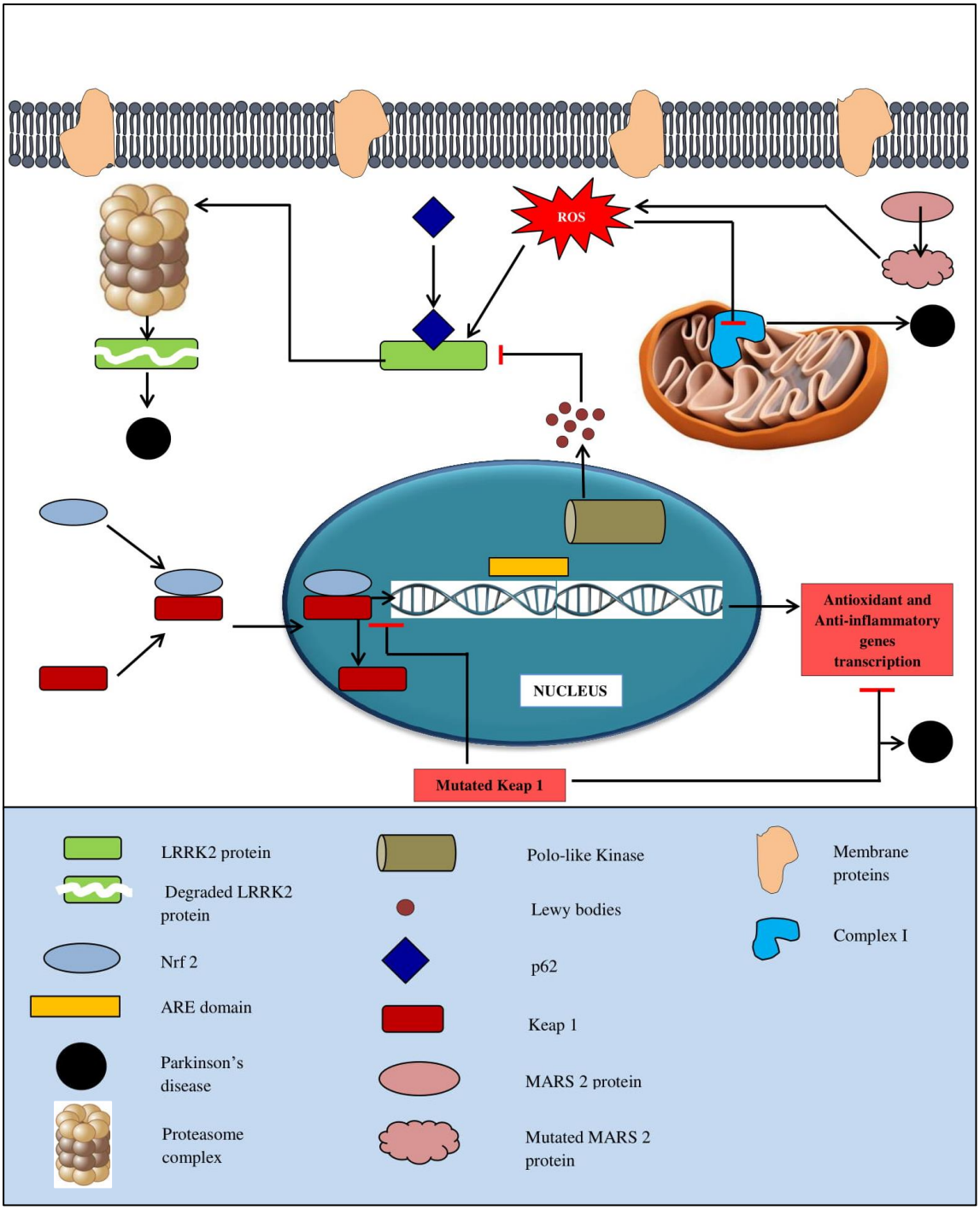


Figure 4.7: Interrelation between the proteins involved in PD

Since decades, natural products have played a crucial role in drug discovery and have led to a revolutionary trend in the pharmacological and medicinal fields. In the present study, we have identified some methylated flavonoids that have potential against the proteins involved in the pathology of PD. For this the correlations between the different target proteins in PD like Methionyl-tRNA synthetase, murine Keap-1, Roco-4 kinase and polo-like kinase were studied. It was found that involvement of all these proteins is directly or indirectly related to LRRK2 gene. All the target proteins inhibit LRRK2 either upstream or downstream of its pathological pathway. Besides that, inhibition of target proteins by phytochemicals might result in the modulation of ROS accumulation; decrease in lewy bodies aggregation; disruption of interaction between Keap-1 and p62 and prevention of degradation of LRRK2. Thus, *in vitro* and *in vivo* studies should be done for the validation of above in silico findings.

List of publications

- Swagata Das, **Prareeta Mahapatra**, Priyanka Kumari. Prem Prakash Kushwaha and Shashank Kumar. (2018). Phytochemicals as Hope for the Treatment of Hepatic and Neuronal Disorders, Phytochemistrty, *In* Volume 2: Pharmacogonosy, Nanomedicine, and Phytochemicals as foes, Eds: Egbuna Chukwuebuka, Shashank Kumar, Ifemeje Jonathan Chinenye, Jaya Vikas Kurhekar, CRC press, USA. (*Communicated)*

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ABSTRACT

Identification of natural inhibitors of proteins involved in the pathology of Parkinson's disease

Name of student : Prareeta Mahapatra Registration number : 16mslsbc15 Degree for which submitted : Masters in Life Sciences Name of supervisor : Dr. Shashank Kumar Name of Department : Biochemistry and Microbial Sciences Name of school : Basic and Applied Sciences

Keywords: Anti-Parkinson products, In silico, Protein targets, Molecular Docking, Drug likeness Property, Phytochemicals.

Parkinson's disease (PD) is a progressive neurodegenerative disorder caused due to the lack of dopamine in the brain. Different drug therapies are available for PD showing excellent efficiency, but most of them are cost intensive and with side effects. All these issues have brought natural products in attention. The present study was designed to identify the potent anti-Parkinson phytochemicals. Proteins that are involved in Parkinson's disease were targeted. In the present study, methylated flavonoids were selected for studies including molecular docking against protein involved in Parkinson's disease such as Murine Keap 1 (5CGJ), brain permeable Polo-like kinase (4I5P), Methionyl tRNA synthetase(1PFU) and Roco-4-kinase(4F0F).To predict the drug-likeness property of the phytochemicals, Lipinski's rules of five, Caco-2, CMC-like rule and MDCK value were used. By prediction of ADME, drug-likeness properties and toxicity properties of the phytochemicals it can be stated that most of the phytochemicals have the potential to cross the Blood Brain Barrier and have good ROS quenching potential also.

Prareeta Mahapatra Dr. Shashank Kumar (Supervisor)

1.1 Parkinson's disease and natural products

Parkinson's disease (PD) is the second most prevalent progressive neurodegenerative disorder which is caused due to the lack of dopamine in the brain and is characterized by tremor, Akinesia, Bradykinesia and postural instability. Mutations in different genes e.g. SNCA, LRRK2, Parkin, PINK2, DJ-1, etc. are found to be involved in PD and these genes can be targeted to treat PD up to an extent (Elbal, et al, 2002). Different drug therapies are available for PD, showing excellent efficiency, but most of them are cost-intensive, with side effects and can only cure the disease symptomatically (only reduction in symptoms). All these issues have brought natural products in attention. Since last decades various phytochemicals are actively used by people to treat many kinds of diseases. Natural products derived from plants have made their own position in the treatment of different neurological disorders. Proper considerations and clinical trials were made on some phytochemicals and clinical tests were made for their validation, mechanism of working and biochemical recovery. Plant-derived molecules have already been examined for their cellular, behavioral and biochemical protection against PD and many other neurological disorders. These phytochemicals are easily available and have no/very poor side effects. Moreover, these also have antioxidant properties. For example terpenoids like geraniol, catalpol, tanshinone and flavonoids like

baicalein and lactones like EGCG etc. are on clinical trials and are proving as potential natural products that can inhibit different causes that lead to PD (Suk, et al, 2005). Natural products have played a very crucial role in drug discovery and have led to a revolution in pharmacology and medicine. Many natural anti-Parkinson drugs have been approved internationally and actively used in its therapy. Many other natural phytochemicals are under clinical trials. New approaches like Computer-aided drug designing (CADD) is being exploited to identify hits, pick leads and optimize drug leads by studying their physicochemical, pharmaceutical and ADMET (absorption, distribution, metabolism, excretion and toxicity) properties for more precise validation of these natural products as antiparkinson drugs. These new approaches can play a critical role in the treatment of PD by identification of leading phytochemicals that can inhibit different proteins involved in the pathology of Parkinson's disease. 1.2 Hypothesis The present study has been designed to identify the active phytochemicals as well as their scientific validation using in silico studies.

2.1 Parkinson's Disease Parkinson's disease is a progressive neurodegenerative disorder which is caused due to the lack of dopamine in the brain and is characterized by tremor, akinesia, bradykinesia and postural instability. The main cause of the disease was discovered to be the fall in the level of dopamine in the brain. Death of dopaminergic neurons in the substantia nigra of the brain leads to decrease in the level of dopamine which in turn results in overstimulation of acetylcholine target neurons. More Acetylcholine induces overstimulation of GABAergic neurons in the substantia nigra and then in the thalamus. As a result, the balance between glutamate and GABA is disrupted which results in abnormal signaling and leads to impaired mobility. Different causes of death of dopaminergic neurons include accumulation of alpha-synuclein fibrils in the substantia nigra part of the brain due to the mutation in the SNCA gene or alpha-synuclein gene (Polymeropoulos, et al, 1997), which leads to proteasomal and lysosomal system dysfunction and reduced mitochondrial activity by affecting its complex I (Keeney, et al, 2006), LRRK2 gene codes for leucine-rich repeat kinase is the only known gene coding for a protein having both kinase and GTPase domain. Mutation in this gene is responsible for the decrease in the GTPase activity and impaired kinase activity which leads to dysfunctioning of presynaptic protein sorting and axonal trafficking, Parkin gene, a part of ubiquitin-ligase complex when homozygously mutated, disrupts the activity of the later which leads to the accumulation of toxic substrates. It leads to the formation of Lewy bodies and finally death of dopaminergic neurons occurs. Other genes involved in PD are DJ-1, PINK-1, transcription factor Nrf-2 etc. (Elbal, et al, 2002). All these proteins are targeted either by synthetic drugs or by drugs obtained from natural products. 2.2 Natural antiparkinson products Natural products have been a great source for treatment of PD. They provide a significant approach on where and how to inhibit different molecular and biochemical causes of PD. The antioxidant medicinal plants are a good source for protection against Parkinson (). These include Brahmi that may improve brain circulation and even protect brain cells from further damage, Cowhage contains Levodopa or L-dopa is administered against Parkinson's disease. (Szego, et al, 2012) Turmeric contains a compound curcumin that is responsible for the disruption of proteins involved in PD and it also prevents their aggregation, Gingko Biloba extract showed neuroprotective and neuro-recovery effects against dopaminergic neuron damage and even damage that affects locomotion.

2.3 History of natural antiparkinson products The concept of natural products has its roots back in 17th and 18th century. Many plant products are used since those days and have been established as potential drugs against PD. Epicatechin-3-gallate (EGCG) in Camelia sinensis (green tea), is the most abundant polyphenol and has anti-inflammatory and neurodegenerative effects. The root extract of Withania somnifera is rich in steroidal lactones (withanone, withaferin, withanolides, and withasomidienone). These were discovered to have potential to inhibit metastasis and Quinone reductase activity. Caffeine, an adenosine 2A receptor antagonist present in coffee beans, inhibits the MPTP induced toxicity by depleting the toxic product 6-ODHA. Ginkgolides and Bilobalides found in Ginkgo biloba have a protective role against ROS production (Smith, et al, 1996) and oxidative stress (DeFeudis, et al, 2000). Baicalein, a flavonoid found in large concentration in Scutellaria baicalensis, attenuates the iron-induced DA–depletion in the substantia nigra and also inhibits the alpha-synuclein aggregation by increasing GSH level (Hamada, et al, 2002). Levodopa was first isolated from the leguminous plant Mucuna pruriens (velvet beans) is an FDA approved drug used for the treatment of PD. Historically natural products have been a rich source of compounds that have a great importance in medicines and therapeutics. Drugs of natural origin can be classified as original natural products, products derived semi-synthetically from natural products or synthetic products based on natural product models. Natural products were examined to have a significant effect on the pathology of Parkinson's disease. The knowledge associated with traditional medicine and use of medicinal plants as potential medicines has led to the isolation of many natural products that have become well-known pharmaceuticals today.

2.4 Importance and advantages of natural antiparkinson products Due to development of resistance to therapeutic drugs search for new antiparkinson drugs and therapies is still a priority goal for the treatment of PD. Natural products considered to be the best source of drugs for past many years as these having no or very poor side-effects, easy availability, and cost-effective. Moreover, natural products have structural and chemical diversity, and there is a substitute option most of the time when one is not available. Hence, natural products are a good source of active therapeutic agents.

2.5 Currently used natural antiparkinson products Several phytochemicals are known to treat or manage PD. Some are discussed in the next section. 2.5.1 Crocin

Crocin, a carotenoid found in flowers, is primarily responsible for the color of saffron. It acts as an antioxidant, as it quenches free radicals, protects cells and tissues against oxidation. Pretreatment of cells with crocin inhibits ROS generation in the dopaminergic terminal and hence prevents the death of dopaminergic neurons. (Zhang, et al, 2015).

Figure 2.1: Inhibition of ROS by Crocin

2.5.2 Geraniol Geraniol is a monoterpenoid and alcohol. It is the primary part of palmarosa oil, rose oil, citronella oil, lemon and much other essential oils (Tiwari, et al, 2009). It acts as an antioxidant and is able to cross the blood-brain barrier. It has cytoprotective characteristics against oxidative stress produced due to various neurotoxins. It helps to restore the membrane potential of mitochondria. Case 1: In the blood-brain barrier, monoamine oxidase-

B is present which is responsible for converting non-toxic MPTP to toxic MPP+ (Rekha, et al, 2013). The administration of geraniol in the early stage of PD can destabilize the enzyme MAO-B, and hence toxin formation can be put to an end (Ben-Shlomo, et al, 2004). Case 2: Geraniol can prevent the aggregation of alpha-synuclein and hence prevents the formation of alpha-synuclein inclusions, which is a major cause of PD and other neurological disorders. (Szego, et al, 2012)

Figure 2.2: Geraniol inhibits MAO-B and α-synuclein aggregation 2.5.3 Safranal Safranal, an organic compound isolated from saffron, is a spice that contains the stigma of crocus flower which is completely responsible for saffron's aroma. It inhibits rotenone-induced cell death (Radad, et al, 2006). Rotenone significantly increases the release of lactate dehydrogenase into the surrounding. It decreases the membrane potential of mitochondria and increases the production of reactive oxygen species, and an anaerobic state of respiration is established (Pan, et al, 2016).

Figure 2.3: Safranal inhibits ROS production

2.5.4 Catalpol Catalpol is an iridoid glycosides, simple monoterpenes with an attached glucose moiety. It is found in the plants that belong to families like Scrophulariaceae, Lamiaceae, and Bignoniaceae, etc. MPP+ is found to be responsible for the opening of (Mitochondrial permeability transition) MPT pore and release of cytochrome c in brain and mitochondria (Iverson, et al, 2004). This is responsible for the mitochondrial destruction (Mao, et al, 2007). Catalpol treatment reduces the activity of MPP+ regarding the opening of MPT pore (Bi, et al, 2008). On the other hand, Ca2+ concentration is also elevated in case of neuronal cells of PD patients, which is also responsible for the opening of MPT pore. Catalpol also restrains the pressure of overloading Ca2+.

Figure 2.4: Catalpol inhibiting trigger compounds for MPT pore opening. 2.5.5 Tanshinone Tanshinone is a diterpenoid and a major lipophilic bioactive compound found in Salvia miltiorrhiza (Family I). It mediates neuroprotective activity against 6-OHDA-induced oxidative stress via an Nrf2-Are pathway (Kaidery, et al, 2013). The transcription factor Nrf2 plays an important role in the induction of cytoprotective genes like those that encode for endogenous antioxidants such as heme oxygenase-1, glutathione cysteine ligase regulatory subunit (GCLC) and glutathione cysteine ligase modulatory subunit (Todorovic, et al, 2016). On the other hand, 6-OHDA is a neurotoxin that is initiated by extracellular auto-oxidation of oxidative products that are generated (Hanrott, et al, 2006). The loss of Nrf2-mediated transcription increases the chances of dopaminergic neurons to undergo oxidative stress (Jakel, et al, 2007). Normally, Nrf2 is regulated post-translationally and constitutively by Keap1, its antagonist (Jing, et al, 2016). The actual function of Nrf2 begins when the cell is exposed to oxidative stress or electrophilic substances. As a result, the Keap1 is modified, detaches from Nrf2, and proceeds into the nucleus and transactivates many target genes. Under resting metabolic conditions, negative regulation of Nrf2 is mediated by Keap1 through ubiquitination. Under conditions of stress, Keap1 is oxidized, and Nrf2 is released, which is stabilized by DJ-1 and translocates to the nucleus via ARE enhancers do activates a range of antioxidant enzymes and hence decreases the oxidative stress (Pan, et al, 2016)

Figure 2.5: Nrf2 released by Keap1, Nrf2 enters nucleus for translation

3.1 In silico, antiparkinson potential screening 3.1.1 Protein preparation Crystal structure of target protein, such as Methionyl tRNA synthetase (1PFU), Roco-4 kinase (4F0F), selective and brain-permeable polo-like kinase 2 (415P), murine keap1 (5CGJ), and were retrieved from Protein Data Bank. All the heteroatoms were removed leaving only the residues of the receptor. Preparation of the target protein with Auto Docking Tool involved the addition of polar hydrogen to the macromolecule, to correct the calculation of partial charge. Finally, Gasteiger charges were calculated for each atom of the macromolecule.

3.1.2 Ligand preparation Literature-based phytochemicals have been used as a ligand for molecular docking. The 3D or 2D structure of phytochemicals and reported inhibitors of particular protein were retrieved from NCBI PubChem in sdf format respective. Open Babel molecule format converter was used for conversion of 2D to 3D conformation, Marvin Sketch software will perform the conversion from sdf to PDB (for docking) and mol (for molecular properties prediction) file. The energy of the ligands was minimized by applying mmff94 force field and optimization of conjugate gradients algorithm was done using PyRx-Python prescription 0.8 (Olson, et al, 2015) for 200 steps.

3.1.2.1 Molecular docking For the docking of different targeted proteins with selected ligands (here the ligands are methylated flavonoids) virtual molecular screening is used. Docking of the ligands and proteins was done in Pyrx software. In Pyrx software, the proteins were first loaded and then converted to macromolecule. The ligand (one at a time) is imported and their energy was minimized by application of mmff94 force field. Then axes, conformations and orientations of the ligands were set in the protein structure by clicking on the vina wizard. Then it was run to obtain the binding affinity (table 4.3). Docking was performed with the targeted protein interface by keeping the points of X, Y and Z dimensions as 25.0000, 25.0000 and 25. 0000 respectively and center grid box values were kept 15.066, 54.587 and 13.5861 for x, y and z centre of Methionyl-tRNA synthetase; -6.6117, 20.2038 and -18.5522 for x, y and z of Roco-4 kinase; 10.2022, 7.0789 and 10.2418 for x, y and z of polo-like kinase; 40.06, -20.46 and -4.9 for x, y and z of Murine keap-1. The grid boxes represent the entire binding site of the protein interface and provide space for the ligand’s binding with respect to all the three axes. Then visualization of the interaction pattern of the complex then formed was done (the protein-ligand complex).

3.1.2.2 ADME Prediction ADME is used as an abbreviation for Absorption, Distribution, Metabolism and Excretion in pharmacology. These four criteria influence the drug levels and drug exposure to tissue and influence the kinetics related to the drug’s performance and pharmacological activity of a compound as a drug. Blood Brain Barrier (BBB), colorectal adenocarcinoma cells (Caco-2), Madin - Darby Canine Kidney cells (MDCK), HIA(Human Intestinal Absorption) and Plasma Protein Binding (PPB) properties were studied using pre - ADMET server.

3.1.3 Drug likeness prediction Number of hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD), Mol Log P, Mol Log S, drug-likeness model score properties were studied using pre- ADMET server.

3.1.4 Toxicity prediction AMES test, Human Ether-ά- go- go gene (HERG inhibition), Carcino rat properties were studied using pre- ADMET sever.

3.1.5 Ligplot analysis Ligplot is the software that generates a 2D diagram showing ligand-protein interaction. It reveals the number of hydrogen bonds and hydrophobic interactions, as well as with which amino acids of the protein the ligand is interacting and the chain number also.

3.1.6 Surface structure analysis In Ligplot 2D diagram, the ligand pocket or active site of the protein cannot be visualised, therefore Pymol was used to visualize the surface structures of the protein and ligand interaction. 3.2 In vitro screening Laboratory techniques: 3.2.1 Anticancer potential screening assay: MTT assay In vitro anticancer potential of samples against various cancer cell lines were performed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cell suspension (100 l) was incubated for 24 h followed by addition of 100 l extracts (100 g/well) and further was incubated for 72 h. MTT solution (10 l) was added to each of the 96 wells, and then plates were wrapped with aluminum foil and incubated at 37◦ C for 4 hour. This leads to the formation of MTT-formazon crystals. Media was removed and 100µl of DMSO was added in each well. Absorbance was measured by plate reader at 590 nm. Controls and samples were assayed in triplicate. The results were shown as mean ± SD.

3.2.2 Antioxidant potential screening assay: DPPH assay The free radical scavenging activity of the samples was measured in vitro by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Appropriate DPPH solution concentration was prepared in methanol followed by addition of 1 ml of the test sample at different concentration. The content was mixed and allowed to stand at measured by recording the absorbance at 517 nm. The percentage scavenging activities (% Inhibition) at different concentrations of the extracts were calculated using the following formula: (%) I= [(Ac−As)/Ac] ×100 Where “I” is inhibition and Ac and As are the absorbance values of the control and the sample, respectively. Three replicates were made for each sample and results were expressed as mean ± SD.

3.2.3 Antibacterial potential screening assay: Disc Diffusion method Antimicrobial activity of test samples against Escherichia coli was determined using Kirby-Bauer disc diffusion method. The inoculum suspensions of bacterial strains were swabbed on the entire surface of LB agar. Sterile 6 mm diameter paper discs (Himedia) saturated with 10 g of phytochemicals (drugs) prepared in DMSO (containing 2 mg extract/disc) were aseptically placed on the upper layer of the inoculated agar surfaces and plates were incubated at 37°C for 24 hours. Antibacterial activity was determined by measuring the diameter of the zone of inhibition (ZOI) surrounding discs. Standard antibiotic discs of Penicillin and Norfloxacin were used as positive control. Discs containing 20 µL DMSO were used as a negative control. The antimicrobial assay was performed in triplicate and results were reported as the average of three replicates (Bauer, et al, 1996).

4.1 In silico antiparkinson’s potential of identified phytochemicals 4.1.1 Molecular drug-likeness property and toxicity prediction of the phytochemicals For the present study 69 phytochemicals (fig 4.1) were selected based on their suitability for Chemistry manufacture

and control (CMC) like rule, Rule of Five. The Chemistry manufacture and control (CMC) like rule, Rule of Five distinguishes between the drug like and non-drug like molecules. These phytochemicals include The ADME property, drug likeness property and toxicity property of the phytochemicals such as Blood brain barrier (BBB) penetration, Caco-2 permeability, Human Intestinal Absorption (HIA), Madin-Darby Canine Kidney cells (MDCK), Pgp inhibition, Plasma Binding Protein (PBP), CMC (Chemistry manufacture and control) like rule, Rule of five, mutagenicity (Ames test), Carcino Rat (CR) and hERG inhibition (hERG-I) were studied in silico.

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ADME includes BBB test, Caco-2 permeability test, HIA test, P-gp inhibition test, MDCK permeability and PPB test. BBB permeability test is done to predict the compounds ability to penetrate into the BBB by calculating the logD/logK value. Compounds showing BBB+ are able to cross the BBB and those showing BBB- are not able to do so. Out of 69 compounds taken as ligands, 12 are BBB+ and rest are BBB- (table 4.1). Caco-2 permeability test is done to validate the suitability of the compounds for oral administration and to predict human intestinal

absorption rate and drug efflux (Breemen, et al, 2005). It measures the in vivo absorption of the drug across the gut wall. HIA test (Human intestinal absorption) provides the information about the compound's absorption into bloodstream (Wessel, et al, 1998). This is important as a drug to be suitable, oral administration; it should be absorbed into the bloodstream. Percentage of HIA is defined as the percentage of drug administered orally that reaches the hepatic portal vein. From the table, we can conclude that more than 70% of each of the ligands reaches the hepatic portal vein and 5 out of 69 have 100% HIA. P-gp means P-glycoprotein is a transport protein molecule (Pillans, et al, 2014). When a drug is actively transported by P-gp then its bioavailability reduces, therefore its inhibition is important (Amin, et al, 2013). Out of 69, 24 are non-inhibitor but rest 45 are inhibitors of P-gp. So, most of the drugs have good bioavailability. MDCK (Madin- Darby canine kidney) cells are the types of cells that show low expression of transporter proteins and low metabolic activity (Irvine, et al, 1999). MDCK permeability is linked to P-gp. It predicts the extent to which the drugs will efflux. PPB (Plasma protein binding) indicates the period of time the drugs will remain in the blood. Drugs interact with the plasma or tissue protein and form complex. It indicates the distribution of drugs. Drugs dynamics is important as only the free drug molecules will interact with the receptors on the targeted proteins. Means more the percentage of binding, poor was the drug. 95% free means 5% is free to actively work against the target. Here none of the ligands shows 100% binding, means some part of the drugs will remain free for the targeted proteins. Drug-likeness property includes CMC-like rule (comprehensive medicinal chemistry) and Rule of five. CMC-like rule defines the drug-like characters of the selected ligands or compounds as compared to the CMC database. It indicates a ligand as qualified or not qualified on the basis of a range. As per CMC database, the qualifying range of log P is -0.4 to 5.6, that for molecular weight is 160 to 480, that for molar refractivity is 40 to 130 and that for total number of atoms is 20-70. Out of 69 only one compound is CMC not qualified (table4.2). Rule of five was given by Chris Lipinski. This rule stated that if a compound violates two or more conditions out of these following conditions like <5 hydrogen bond donors, molecular mass <500, calculated log P value <5, Sum of donors and acceptors should be <10, then they are said to have poor absorption with poor permeability. In this case, all the 69 ligands are suitable and passed the rule of five (table4.2). Ames test was developed by Bruce Ames, used to examine whether the compound taken as ligand is able to cause mutations in the DNA of the test organism or not. A positive test indicates that the compound is mutagenic and may act as a carcinogen (as most of the cancers are related to mutation). But there is no complete association between a compound being mutagenic and carcinogenic always. (False positive and false negative are known). Here out of 69, 3 are non-mutagen and rest are mutagens (table4.2). Carcino rat test signifies that a given compound is able to produce carcinogenic effect in rats or not (Wanibuchi, H., et al, 2004). Out of 69, only one is Carcino rat negative, rest others are positive (table4.2). hERG expands as human ether-a-go-go-related gene. It is involved in cardiac repolarization and encodes for the gene involved in inward rectifying voltage-gated potassium channels in the heart. Inhibition of hERG prolongs the QT interval of the cardiac cycle and this leads to the potential fatal ventricular tachyarrhythmia causing cell death of the test organism. Not all compounds that exhibit hERG inhibition will proceed to cardiotoxicity but still, its inhibition is a sensitive measure to cross checks the compounds. Out of 69, 34 exhibits low risk whereas rest show medium risk (table4.2).

Table4.1: ADME properties of the ligands and standard inhibitors

STANDARDS ADME PROPERTY Standards BBB Caco-2 HIA MDCK Pgp(I) PPB 1 BBB+ 12.4418 91.7252 7.62836 Non-inhibitor 95.59209 2 BBB- 22.5596 93.50045 0.0822323 Inhibitor 89.86518 3 BBB+ 23.037 99.28634 61.0892 Non-inhibitor 100 4 BBB+ 19.5023 98.34936 0.0443177 Inhibitor 100

LIGANDS ADME PROPERTY Ligands BBB Caco-2 HIA MDCK Pgp(I) PPB 1 BBB- 7.02526 88.18826 23.8531 Non-inhibitor 90.16013 2 BBB- 9.32409 88.18001 27.7465 Non-inhibitor 90.72856 3 BBB- 4.93945 78.34267 21.9216 Non-inhibitor 85.3466 4 BBB- 10.8691 78.33157 27.782 Non-inhibitor 90.00934 5 BBB- 37.3659 95.99601 182.247 Non-inhibitor 86.77201 6 BBB- 15.6876 93.59491 42.2482 Inhibitor 89.12084 7 BBB- 5.3654 87.82133 83.9141 Non-inhibitor 87.15776 8 BBB- 5.08813 87.82152 7.10806 Non-inhibitor 81.90835 9 BBB- 6.3241 76.28319 2.76593 Inhibitor 86.90252 10 BBB- 9.63521 76.304 35.2941 Non-inhibitor 79.61837 11 BBB+ 55.3919 97.92488 2.91282 Non-inhibitor 89.23074 12 BBB- 33.065 96.48635 1.02959 Inhibitor 87.41127 13 BBB- 37.5234 96.48551 8.29032 Non-inhibitor 87.267 14 BBB- 37.5249 96.48879 5.85056 Inhibitor 87.33959 15 BBB- 9.12274 93.45203 33.6279 Inhibitor 86.59456 16 BBB- 3.1597 86.80217 0.584809 Non-inhibitor 79.07711 17 BBB- 7.6456 86.80483 10.0304 Non-inhibitor 78.7669 18 BBB- 8.20745 86.799 1.1177 Inhibitor 83.82541 19 BBB- 6.65579 86.79903 31.0397 Non-inhibitor 83.98422 20 BBB- 5.79119 73.19857 0.347829 Non-inhibitor 76.5246 21 BBB- 53.769 98.44068 0.43586 Inhibitor 88.11521 22 BBB- 54.9919 98.44068 2.46246 Inhibitor 88.01683 23 BBB- 30.4875 96.80767 0.140155 Inhibitor 85.89788 24 BBB- 24.7609 96.80845 7.72373 Non-inhibitor 80.41033 25 BBB- 25.2665 96.80636 7.02672 Non-inhibitor 81.4828 26 BBB- 30.4903 96.80845 32.6907 Non-inhibitor 81.57822 27 BBB- 36.4848 96.80636 1.12492 Inhibitor 86.66454 28 BBB- 36.4931 96.80987 4.19207 Inhibitor 86.60702 29 BBB- 9.13705 93.45634 3.1826 Non-inhibitor 82.20566 30 BBB- 36.4848 96.80636 1.12492 Inhibitor 86.66454 31 BBB- 7.33774 93.79605 5.12038 Inhibitor 82.58061 32 BBB- 53.4985 98.88618 11.6373 Inhibitor 83.27144 33 BBB- 54.9919 98.44068 2.46246 Inhibitor 88.01683 34 BBB- 51.2863 98.88618 43.6237 Inhibitor 82.46623 35 BBB- 38.2247 96.79591 7.04255 Non-inhibitor 78.62973 36 BBB- 31.6501 96.79433 7.00188 Inhibitor 80.58084 37 BBB- 43.1616 96.79536 0.286843 Inhibitor 84.80384 38 BBB- 54.0214 99.07504 0.0669953 Inhibitor 84.85934 39 BBB- 41.2261 96.79537 0.10887 Inhibitor 84.57317 40 BBB- 54.0214 99.07504 0.0669953 Inhibitor 84.85934 41 BBB- 41.2216 96.79375 0.0863988 Inhibitor 84.77709 42 BBB- 41.2216 96.79375 0.0863988 Inhibitor 84.77709 43 BBB- 53.3736 98.84629 0.0913785 Inhibitor 87.55638 44 BBB- 47.3048 99.07504 0.117108 Inhibitor 78.33853 45 BBB- 52.7895 99.07504 0.965244 Inhibitor 77.96442 46 BBB- 38.3489 96.39352 0.34293 Inhibitor 76.81297 47 BBB- 54.2909 99.07504 0.506149 Inhibitor 79.99046 48 BBB- 53.6117 99.07504 35.8771 Non-inhibitor 79.28555 49 BBB- 41.0259 96.39538 0.0938047 Inhibitor 78.08129 50 BBB- 42.0076 95.6224 0.0571157 Inhibitor 71.83945 51 BBB- 54.2805 98.02333 0.571395 Inhibitor 86.17298 52 BBB- 37.0482 96.39311 0.181877 Inhibitor 76.27377 53 BBB- 41.0379 96.90329 4.80685 Inhibitor 89.78122 54 BBB+ 55.1765 100 52.6243 Inhibitor 91.74429 55 BBB+ 47.4485 96.43149 0.0851212 Inhibitor 90.31974 56 BBB- 42.4072 97.03145 0.0464288 Inhibitor 86.36673 57 BBB- 43.0811 96.9028 0.0571744 Inhibitor 87.17774 58 BBB- 44.461 96.49206 0.18686 Non-inhibitor 88.27599 59 BBB- 41.4489 96.35932 0.127776 Inhibitor 85.94318 60 BBB+ 54.057 98.89852 0.0674751 Inhibitor 85.16708 61 BBB+ 51.2529 98.86539 0.0694854 Non-inhibitor 86.8745 62 BBB+

55.2708 98.86539 0.218232 Inhibitor 86.41294 63 BBB+ 53.628 98.86539 0.629079 Inhibitor 87.51192 64 BBB+ 51.2529 98.86539 0.0694854 Non-inhibitor 86.8745 65 BBB- 7.00371 87.19291 24.4257 Non-inhibitor 96.79283 66 BBB+ 56.6935 100 45.9875 Inhibitor 93.22826 67 BBB+ 56.6128 100 40.5913 Inhibitor 94.9859 68 BBB+ 56.0673 100 51.765 Inhibitor 95.09543 69 BBB+ 56.4306 100 41.1392 Inhibitor 94.64093

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Table4.2: Drug likeness and toxicity properties of the ligands and the standard inhibitors. Standards Drug-likeness property Toxicity property CMC like rule Rule of five Ames test Carcino rat hERG Inhibition 1 Q Suitable M Negative MR 2 Q Suitable M Negative MR 3 Q Suitable M Positive LR 4 NQ Suitable NM Negative LR

Ligand Drug-likeness property Toxicity property CMC Like Rule Rule of Five Ames test Carcino Rat hERG Inhibition 1 Q Suitable M Positive MR 2 Q Suitable M Positive MR 3 Q Suitable M Positive MR 4 Q Suitable M Positive MR 5 Q Suitable M Positive MR 6 Q Suitable M Positive MR 7 Q Suitable M Positive MR 8 Q Suitable M Positive MR 9 Q Suitable M Positive LR 10 Q Suitable M Positive LR 11 Q Suitable M Positive LR 12 Q Suitable M Positive LR 13 Q Suitable M Positive LR 14 Q Suitable M Positive LR 15 Q Suitable M Positive LR 16 Q Suitable M Positive LR 17 Q Suitable M Positive LR 18 Q Suitable M Positive LR 19 Q Suitable M Positive LR 20 Q Suitable NM Positive LR 21 Q Suitable M Positive MR 22 Q Suitable M Positive MR 23 Q Suitable M Positive MR 24 Q Suitable M Positive MR 25 Q Suitable M Positive MR 26 Q Suitable M Positive MR 27 Q Suitable M Positive MR 28 Q Suitable M Positive MR 29 Q Suitable NM Positive MR 30 Q Suitable M Positive MR 31 Q Suitable M Positive LR 32 Q Suitable M Positive MR 33 Q Suitable M Positive MR 34 Q Suitable M Positive MR 35 Q Suitable M Positive LR 36 Q Suitable M Positive LR 37 Q Suitable M Positive LR 38 Q Suitable M Positive LR 39 Q Suitable M Positive LR 40 Q Suitable M Positive LR 41 Q Suitable M Positive LR 42 Q Suitable M Positive LR 43 Q Suitable M Positive LR 44 Q Suitable M Positive LR 45 Q Suitable M Positive LR 46 Q Suitable M Positive LR 47 Q Suitable M Positive LR 48 Q Suitable M Positive LR 49 Q Suitable M Positive LR 50 Q Suitable M Positive LR 51 Q Suitable M Positive MR 52 Q Suitable M Positive LR 53 Q Suitable M Positive LR 54 Q Suitable M Positive MR 55 Q Suitable NM Positive MR 56 Q Suitable M Positive MR 57 Q Suitable M Positive MR 58 Q Suitable M Positive MR 59 Q Suitable M Positive LR 60 Q Suitable M Positive LR 61 Q Suitable M Positive MR 62 Q Suitable M Positive MR 63 Q Suitable M Positive MR 64 Q Suitable M Positive LR 65 Q Suitable M Positive MR 66 NQ Suitable M Positive MR 67 Q Suitable M Positive MR 68 Q Suitable M Positive MR 69 Q Suitable M Negative MR

Note: CLR, CMC like rule, RF, Rule of five, AT, Ames test, hERG-I, human ether-a-go-go related gene, CR, Carcino rat, M, Mutagen, NM, Non-mutagen, Q, Qualified, NQ, Not qualified, N-(5-methyl-1H-pyrazol-3-yl)-2-phenylquinazolin-4-amine,4-[4-[[4-chloro-3-(trifluoromethyl)phenyl] carb amoylamino]phenoxy]-N-methylpyridine-2-carboxamide,2-methyl-5-propan-2-ylcyclohexa-2,5-diene -1,4-dione,2-[[4-[carboxymethyl-(4-methoxyphenyl)sulfonylamino] naphthalen-1-yl]-(methoxyphenyl) -sulfonyl- amino] 4.1.2 Ligand-Protein Binding analysis The ligands were docked against the targeted proteins for the prediction of binding score. Table 4.3 shows the result of molecular docking studies in the form of binding energy score. The

score reveals the highest and lowest binding score of different ligands with different target proteins. It also shows the binding score of the standard inhibitors of the respective proteins (table 4.3). More negative the binding energy score is, more affinity the ligand has for the respective protein. For 1PFU (methionyl-tRNA synthetase), two ligands (68 and 69) showed better binding energy (-10.4 and -10.3 Kcal/mol respectively) as compared to the standard inhibitor (-10.1Kcal/mol). For 4F0F (Roco-4 Kinase), 8 ligands show more negative binding energy (ranging from -7.1 to -8.6 Kcal/mol) as compared to the standard (-7 Kcal/mol). For 4I5P (Brain permeable polo-like Kinase 2), all the ligands show more negative binding energy (ranging from -7 to -11.4 Kcal/mol) as compared to the standard (-6.7 Kcal/mol) and for 5CGJ (Crystal structure of murine keap-1), 58 ligands show more negative binding energy (ranging from -4.5 to -7.9 Kcal/mol). Ligands 66, 67, 68 and 69 depicted higher affinities towards 1PFU, 4F0F and 4I5P as indicated by more negative binding energy values (-7 to -11.4 Kcal/mol) Note: MF- methylated flavonoids

4.1.3 Ligplot analysis

Ligplot is the software that generates a 2D diagram showing ligand-protein interaction. It reveals the number of hydrogen bonds and hydrophobic interactions, as well as with which amino acids of the protein the ligand is interacting and the chain number also. Pattern of interaction between ligand and protein is shown in figure 4.2. Position and name of amino acids involved in the interaction pattern such as hydrogen bonding and hydrophobic interaction are given in table 4.4.

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Figure 4.2: Ligplot showing amino acids of the target protein involved in interaction with ligand:(1)Methionyl tRNA synthetase and ligand 1, (2)Methionyl tRNA synthetase and ligand 2, (3)Methionyl tRNA synthetase and ligand 3, (4)Methionyl tRNA synthetase and ligand 4, (5) Methionyl tRNA synthetase and ligand 21, (6)Methionyl tRNA synthetase and ligand 54, (7) Methionyl tRNA synthetase and ligand 65, (8)Methionyl tRNA synthetase and ligand 66, (9) Methionyl tRNA synthetase and ligand 68, (10)Methionyl tRNA synthetase and ligand 69(11) Roco-4 Kinase and ligand 1, (12)Roco-4 Kinase and ligand 2, (13)Roco-4 Kinase and ligand 5, (14)Roco-4 Kinase and ligand 14, (15)Roco-4 Kinase and ligand 18, (16)Roco-4 Kinase and ligand 65, (17)Roco-4 Kinase and ligand 66, (18)Roco-4 Kinase and ligand 67, (19)Roco-4 Kinase and ligand 68, (20)Roco-4 Kinase and ligand 69, :(21)Brain permeable polo-like kinase and ligand 1, (22)Brain permeable polo-like kinase and ligand 2, (23)Brain permeable polo-like kinase and ligand 3, (24)Brain permeable polo-like kinase and ligand 5, (25)Brain permeable polo-like kinase and ligand 8, (26)Brain permeable polo-like kinase and ligand 65, (27)Brain permeable polo-like kinase and ligand 66, (28)Brain permeable polo-like kinase and ligand 67, (29)Brain permeable polo-like kinase and ligand 68, (30)Brain permeable polo-like kinase and ligand 69, (31)Murine Keap 1 and ligand 1, (32)Murine Keap 1 and ligand 4, (33)Murine Keap 1 and ligand 5, (34)Murine Keap 1 and ligand 6, (35)Murine Keap 1 and ligand 7, (36)Murine Keap 1 and ligand 8, (37)Murine Keap 1 and ligand 10, (38)Murine Keap 1 and ligand 16, (39) Murine Keap 1 and ligand 17, (40)Murine Keap 1 and ligand 24.

Table 4.4: List of ligands exhibiting hydrogen bonds and hydrophobic interactions

Complex name Residues involved in hydrophobic interactions Hydrogen bonding residues

1PFU-ligand 1 Met 290, Lys 271, Ile 313, Glu 286, Val 256, Ala269, Asp 381, Phe 382, Val 270, Leu 248, Tyr 253, Phe 317, Leu 370, Gly 371

Met 318 1PFU-ligand 2 Met 290, Phe 382, Val 256, Tyr 253, Phe 317, Leu 370, Ala269, Arg 381, Val 229, Glu316, Gly 321

Glu 286, Lys 271, Met 318, Thr 315 1PFU-ligand 3 Val 299, Thr 315, Ala 269, Leu 370, Phe 382, Val 286, Tyr 253, Phe 317, Gly 250, Gly 321, Leu 248

Met 318 1PFU-ligand 4 Gly 250, Gly 321, Leu 248, Phe 317, Ala 269, Leu 270, Phe 382

Asn 322, Glu 316, Met 318, Tyr 253 1PFU-ligand 21 Met 290, Glu 286, Val 299, Asp 381, Lys 271, Ile 313, Phe 382, Val 256, Tyr 253, Phe 317, Met 318, Leu 248 Gly 321

Thr 315 1PFU-ligand 54 Met 290, Asp 381, Glu 286, Val 299, Ala 380, Thr 315, Tyr 253, Phe 382, Ala 269, Gly 321, Met 318, Leu 370, Phe 317

1PFU-ligand 65 Met 290, Ile 313, Lys 271, Val 299, Phe 382, Thr 315, Ala 269, Val 256, Tyr 253, Val 270, Gly 321, Leu 370, Phe 317

Met 318 1PFU-ligand 66 Leu 354, Leu 298, Ile 293, Val 299, Val 289, Asp 381, Val 299, Ala 380, Met 290, Glu 286, Phe 382, Lys 271, Ile 313, Thr 315

1PFU-ligand 68 Ala 380, Glu 286, Asp 381, Met 290, Ile 313, Val 299, Lys 271, Val 256, Phe 382, Leu 248, Ala 269, Phe 317, Gly 321, Tyr 253, Met 318, Leu 370

1PFU-ligand 69 Glu 286, Asp 381, Val 256, Val 299, Lys 271, Leu 370, Phe 382, Glu 316, Leu 248, Ala 269, Tyr 253, Phe 317, Met 318

Thr 315 4F0F-ligand 1 Val 1040, Phe 1161, Asp 1112, Pro 1158, Lys 1034

4F0F-ligand 2 Ala 1053, Val 1040, Phe 1161, Gly 1111, Asp 1112, Pro 1158

4F0F-ligand 5 His 1115, Pro 1158, Lys 1034, Val 1040, Phe 1161, Phe 1107, Lys 1055, Val 1108, Ala 1053

4F0F-ligand 14 Lys 1055, Met 1105, Ala 1053, Asp 1177, Val 1040, Phe 1161, Gly 1111, Pro 1158

4F0F-ligand 18 Phe 1107, Glu 1106, Ala 1053, Gly 1111, Phe 1161, Val 1040, Asp 1177, Pro 1158, Asp 1112, Lys 1034

4F0F-ligand 65 Phe 1161, Val 1040, Lys 1034

4F0F-ligand 66 Phe 1161, Val 1108, Asp 1112, Val 1040, Gly 1111, Lys 1034

4F0F-ligand 67 Val 1040, Val 1108, Lys 1055, Phe 1107, Ala 1053, Phe 1161, Asp 1112, Lys 1034, Pro 1158

4F0F-ligand 68 Val 1108, Phe 1161, Ala 1053, Lys 1055, Phe 1107, Lys 1055, Val 1040, Asp 1112, Lys 1034, Pro1158

4F0F-ligand 69 Phe 1107, Val 1140, Val 1108, Ala 1053, Gly 1111, Phe 1161, Asp 1112, Lys 1034

4I5P-ligand 1

Asp 223, Leu 159, Phe 212, Val 143, Ala 109, Tyr 161, Leu 88

Lys 111, Cys 162 4I5P-ligand 2 Leu 88, Tyr 161, Phe 212, Glu 160, Ala 109, Leu 159, Val 143, Asp 223

Cys 162, Arg 165 4I5P-ligand 3 Val 143, Leu 159, Gly 91, Phe 212, Ala 109, Tyr 161, Leu 88

Glu 160, Lys 111, Cys 162 4I5P-ligand 5 Asp 223, Leu 159, Val 143, Phe 212, Cys 162, Tyr 161, Arg 165, Ala 109, Leu 88

Lys 111 4I5P-ligand 8 Leu 159, Asp 223, Val 143, Gly 91, Phe 212, Ala 109, Leu 88, Ser 163

Glu 60, Lys 111, Cys 161 4I5P-ligand 65 Tyr 161, Ala 109, Asp 223, Leu 159, Phe 212, Lys 111, Val 143

Leu 88, Cys 162 4I5P-ligand 66 Leu 88, Arg 165, Tyr 161, Glu 160, Ala 109, Phe 212, Asp 223, Val 143, Leu 159

Cys 162 4I5P-ligand 67 Glu 160, Leu 159, Lys 111, Asp 223, Phe 212, Cys 96, Ala 189, Leu 88, Tyr 161, Cys 162

4I5P-ligand 68 Val 143, Leu 159, Lys 111, Glu 168, Asp 223, Phe 212, Ala 109, Tyr 161, Leu 88, Cys 162

4I5P-ligand 69 Val 143, Asp 223, Leu 159, Phe 212, Lys 111, Glu 160, Tyr 161, Leu 88, Ala 109, Cys 162

5CGJ-ligand 1 Asn 382, Phe 577, Tyr 334, Ser 363, Ala 556, Arg 415, Gly 509, Gly 462, Gly 364

5CGJ-ligand 4 Asn 414, Arg 380, Asn 382, Gly364, Gln 530, Gly 509, Tyr 525

Ala 556, Arg 415, Ser 555

5CGJ-ligand 5 Asn 414, Gly 364, Arg 380, Ser 363, Ser 508, Ala 556, Ser 555, Tyr 525

Arg 415, Asn382 5CGJ-ligand 6 Ser 508, Tyr 525, Ser 602, Tyr 334, Tyr 572, Tyr 334, Phe 577

Ser 363, Ser 555, Arg 415, Asn382 5CGJ- ligand 7 Tyr 525, Tyr 572, Gln 530, Ala 556, Gly 364, Arg 415

Asn 414, Ser 363, Ser 555, Arg415, Asn382

5CGJ-ligand 8

Arg 415, Arg 380, Ala 556, Asn 382, Tyr 572, Ser 602, Tyr 325, Glu 530

Asn 414, Ser 363, Ser 555 5CGJ-ligand 10 Asn 382, Arg 380, Gly 364, Arg 415, Gly 603, Ala 556, Tyr 572

Asn 414, Ser 363, Ser 555 5CGJ-ligand 16 Arg 380 , Asn 382, Arg 415, Asn 414, Ala 556, Gln 530, Tyr 572

Ser 363, Ser 555 5CGJ-ligand 17 Asn 382, Arg 380, Tyr 572, Gln 530, Gly 603, Ala 556, Gly 364

Asn 414, Ser 363, Ser 555 5CGJ-ligand 24 Asn 382, Arg 380, Asn 414, Gly 683 Ala 556, Ser 602, Gly 364, Tyr 572

Arg 415, Ser 363, Ser 555

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(13)

(14)

Figure 4.3: Surface structure of protein and ligand (best 4 of each protein) (1)Methionyl tRNA synthetase and ligand 65, (2)Methionyl tRNA synthetase and ligand 66, (3)Methionyl tRNA synthetase and ligand 68, (4)Methionyl tRNA synthetase and ligand 69, (5)Roco-4 Kinase and ligand 1, (6)Roco-4 Kinase and ligand 66, (7)Roco-4 Kinase and ligand 67, (8)Roco-4 Kinase and ligand 69,(9)Brain permeable polo-like kinase and ligand 66, (10)Brain permeable polo-like kinase and ligand 67, (11)Brain permeable polo-like kinase and ligand 68, (12)Brain permeable polo-like kinase and ligand 69, (13)Murine Keap 1 and ligand 1, (14)Murine Keap 1 and ligand 6, (15)Murine Keap 1 and ligand 8, (16)Murine Keap 1 and ligand 10.

4.2 Results of laboratory techniques 4.2.1 Anticancer potential screening assay: MTT assay

To access the antiproliferative activity of compound 001, the colon cancer cell lines (HT29) were used using MTT assay and the results are shown in figure 4.4. The result showed that 001 exhibited moderate anticancer efficacy at test concentrations (0.5-0.04 mg/ml). About 55% growth inhibition activity was observed at higher test concentration (0.04 mg/ml) against lung cancer cell line.

Figure 4.4: Viability of the cells decreases with increasing concentration of compound 001. 4.2.2 Antioxidant potential screening assay: DPPH radical scavenging activity. Result showed that the antioxidant activity increased with increasing concentration of the sample (Figure 4.5). The radical scavenging activity of triphenylphosphineruthenium was found to be 75-80% at different test concentration (1–20 l/mL). It was found higher at higher concentrations (100 l/ mL) (Mishra et al., 2013)

Figure 4.5: Percentage scavenging activity of compound 001 4.2.3 Antibacterial potential screening assay: Disc diffusion method

(c)

(a)

(b)

In this experiment norfloxacin and penicillin were used as standards. It was observed that no colonies emerged near the antibiotic norfloxacin, a few colonies grew after a rim of few millimetres and there was no effect of the antibiotic a few centimetres away it. In case of penicillin, inhibition was less as compared to norfloxacin. But in case of compound A, there was no significant inhibition of growing colonies.

(c)

Figure 4.6: Antibacterial activity by disc diffusion assay of (a) norfloxacin, (b) penicillin and (c) compound A.

Discussion

The different proteins involved in PD either for their upregulation or downregulation are found to be interconnected to each other. The imbalance in the ROS concentration leads to several pathophysiological conditions like diabetes, cancer and many neurodegenerative diseases like Parkinson’s disease (Barja, G., et al, 2004) Alzheimer etc. So to protect its organelles, cells use different endogenous enzymatic and non-enzymatic pathways to inhibit ROS production by the production of antioxidant molecules like peroxidase, ubiquinone (ubiquitin proteasome complex) etc. Methionyl tRNA synthetase is an enzyme found in the cytoplasm that charge tRNA with the help of their cognate amino acids. Its function includes methionine ligase activity, tRNA binding, nucleotide binding, ATP binding, RNA binding etc. MARS 2, a gene in human codes for Methionyl tRNA synthetase (it is among those genes that are translocated inside the nucleus). Mutation in this gene leads to inhibition of complex I and increases the concentration of ROS (Bayat, V., et al, 2012). There are no studies available regarding the inhibitors of Methionyl tRNA synthetase in humans. Till date, there is no identification of such compounds (whereas inhibitors of Methionyl tRNA synthetase of gram-positive bacteria, malarial parasites and other bacteria are known). In this study, we reported that methylated flavonoids numbered 2-(3,4-dimethylphenyl)-5,7-dimethyl-4H-chromen-4-one and (2S)-2-(3,4-dimethylphenyl)-5,6,7-trimethyl-3,4-dihydro-2H-1-benzopyran-4-one showed

better potential against the protein Methionyl tRNA Synthetase as compared to the standards. They depicted 102.9% and 101.9% binding affinity respectively as compared to the standards.

Roco-4 Kinase is a nuclear protein which is 47% similar to LRRK2. It is different from it in the aspect of its isolation as a stable and soluble protein and it is used for the in vivo and in vitro study of the activity of LRRK2 in different diseased conditions like PD, as like LRRK2 it is also inhibited by LRRK2 inhibitors. Inhibitors of LRRK2 are known (they can also inhibit Roco-4 Kinase). By the help of kinase focused set screening, a potential novel drug has been discovered against Parkinson's i.e. 4-ethoxy-7H-pyrrolo[2,3-d]pyrimidin-2-amine (Ding, X., et al, 2018). It was able to penetrate into CNS and in vivo studies revealed that significant level of inhibition of Ser 935 phosphorylation is achieved by its administration in rat and mouse models. In this study we reported that methylated flavonoids numbered 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-5,6,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-3,5,7,8-tetramethyl-4H-chromen-4-one, 2-(3,4-dimethylphenyl)-5,7-dimethyl-4H-chromen-4-one and (2S)-2-(3,4-dimethylphenyl)-5,6,7-trimethyl-3,4-dihydro-2H-1-benzopyran-4-one showed better potential against the protein Roco-4 Kinase as compared to the standards. They depicted 108.57%, 122.85%, 114.28%, 105.71% and 117.14% binding affinity respectively as compared to the standards.

Polo-like Kinase 2(PLK) is a protein found in the nucleus and belongs to serine/threonine kinase family having an N-terminal catalytic domain. Out of 5 discovered PLKs, PLK 2 and PLK 3 directly phosphorylate α-synuclein at Ser 129. The overexpression of PLK2 and PLK3 regulates α-synuclein phosphorylation at Ser 129(Mbefo et al, 2010). Phosphorylation of α-synuclein at Ser 129 results in the accumulation of α-synuclein filaments, then fibrils and then ultimately inclusions or Lewy bodies. This raises the chances of mutation in the kinase domain of LRRK2 which again increases the phosphorylation of α-synuclein (Hayashita-Kinoh, H., et al, 2006). In studies, it has been proved that LRRK2 proteins are found in the periphery of 10-80% of Lewy bodies. (Anderson et al, 2006). Inhibitors of PLK 2 are known. Recent studies have been done on PLK 2 inhibitors based on the tetrahydropteridin chemical scaffold and according to this study two compounds numbered C2 and C21 could be promising PLK 2 inhibitors (Zhan, M.M., et al, 2018). Another study revealed that PLK 2 inhibition is a strong CNS pharmacological target that does not cause genotoxicity (chromosomal damage) and at doses and exposures to ELN582646 can inhibit the same by degradation of the particular protein (Fitzerald, K., et al, 2013). In this study we reported that methylated flavonoids numbered 66, 67, 68 and 69 showed better potential against the protein Roco-4 Kinase as compared to the standards. They depicted 168.65%, 170.14%, 158.2% and 162.68% binding affinity respectively as compared to the standards.

In Erk- Nrf2 pathway, the activation of Erk is required for the activation of Nrf2. The role of Nrf2 in Erk- Nrf2 pathway is the transcription of different genes involved in the production of antioxidant molecules. Nrf2 remains bound to Keap1 (in the cytoplasm). It is required for the transport of Nrf2 factor from outside the cell into the cytoplasm. In oxidative stress condition Keap1 gets modified, detaches itself from Nrf2 and then Nrf2 is released, stabilized by DJ-1 and enters into the nucleus by the help of ARE enhancers and transcription of above mentioned genes takes place. Inhibitors are known. Keap 1 inhibition can be in two different

stages, first while it is outside the cell and second when it reaches the cytoplasm. Outside the cell Nrf2 requires Keap1 for its transport into the cytoplasm (as an anchor protein under stress condition). 1,4-Naphthoquinone (NQ) treatment inhibited mitogen-induced proliferation of lymphocytes via glutathionylation of Keap1. It increases total protein S-thionylation. Molecular docking studies revealed that NQ can disrupt Keap1/ Nrf2 interaction as it directly binds to the binding site of Nrf2 on Keap1 protein and inhibits the entry of Nrf2 into the cytoplasm (Gambhir, L., et al, 2014). But if inhibition occurs after the transport of Nrf2 inside the cytoplasm then only Nrf2 was able to enter the nucleus. Therefore Keap 1 is meant to be degraded. miR200, a regulatory mRNA controls the expression of Keap1 gene. It inhibits Keap1 3'-Untranslated region and results in its degradation (Eades, G., et al, 2011). In this study we reported that methylated flavonoids numbered 1, 6, 8 and 10 showed better potential against the protein Keap 1 as compared to the standards. They depicted 172.09%, 183.72%, 162.79% and 174.41% binding affinity respectively as compared to the standards. Overall the inhibition of Methionyl tRNA synthetase gene i.e. MARS2 leads to the production of ROS. For the suppression of ROS, Nrf2 should transcribe the anti-oxidant genes which are again inhibited by the attachment of Keap1 to Nrf2 factor. Keap1 is associated with p62 (autophagic receptor) is responsible for the LRRK2 degradation as well as its analog Roco-4 Kinase. LRRK2 is also mutated by the Lewy bodies which are formed due to the aggregation of the α-synuclein protein. Phosphorylation of α-synuclein is due to the overexpression of PLK 2 and 3. All the proteins associated with Parkinson's disease are interrelated either by stimulating or inhibiting each other.

Figure 4.7: Interrelation between the proteins involved in PD

List of publications Since decades, natural products have played a crucial role in drug discovery and have led to a revolutionary trend in the pharmacological and medicinal fields. In the present study, we have identified some methylated flavonoids that have potential against the proteins involved in the pathology of PD. For this the correlations between the different target proteins in PD like Methionyl-tRNA synthetase, murine Keap-1, Roco-4 kinase and polo-like kinase were studied. It was found that involvement of all these proteins is directly or indirectly related to LRRK2 gene. All the target proteins inhibit LRRK2 either upstream or downstream of its pathological pathway. Besides that, inhibition of target proteins by phytochemicals might result in the modulation of ROS accumulation; decrease in lewy bodies aggregation; disruption of interaction between Keap-1 and p62 and prevention of degradation of LRRK2. Thus, in vitro and in vivo studies should be done for the validation of above in silico findings.

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