In silico analysis of biomolecules for LRRK2 gene and its clinical relevance

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Submitted by

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CERTIFICATE

This is to certify that the M. Tech. dissertation entitled "***In silico* analysis of biomolecules for LRRK2 gene and its clinical relevance**", submitted by **Ankita Yadav (2K14/BIO/02)** in the partial fulfillment of the requirements for the award of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance. The information and data enclosed in this dissertation is original and has not been submitted elsewhere for honoring of any other degree.

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I declare that my major project entitled **"*In silico* analysis of biomolecules for LRRK2 gene and its clinical relevance"**, submitted to Department of Biotechnology, Delhi Technological University as a result of the work carried out by me at "Molecular Neuroscience and Functional Genomics Laboratory", Department of Biotechnology, as Major project.

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Ankita Yadav
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LIST OF ABBREVIATIONS

S.NO.	ABBREVIATION	FULL FORM
1.	PD	Parkinson's disease
2	LB	Lewy Bodies
3.	LRRK2	Leucine Rich Repeat serine/threonine protein Kinase 2
4	SNCA	Synuclein-alpha
5	MAPKKK	Mitogen-activated protein kinase kinase kinase
6	PDB	Protein data bank
7	BLAST	Basic Local Alignment Search Tool
8	MEGA	Molecular Evolutionary Genetics Analysis

In-silico analysis of biomolecules for LRRK2 gene and its clinical relevance

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ABSTRACT

Millions of individuals worldwide are suffering from Parkinson's disease (PD) which is one of the most common neurodegenerative diseases. Clinically characterization of PD can be shown by rigidity, slow movement, rest tremor etc. On gradual progression of the disease, non-motor symptoms such as anxiety, dementia, constipation and depression can be observed. Mutations in these proteins like Parkin, α-synuclein, Leucine-Rich Repeat Kinase 2(LRRK2), PTEN-Induced Kinase (PINK1) can result in development of PD. Lewy bodies [LB] which is one of the major features of PD consists mainly of aggregates of α-synuclein. Numerous inhibitors of LRRK2 kinase are being identified, which can show protective behavior against LRRK2-induced degeneration of neurons. Thus LRRK2 kinase inhibition seems to be a potential new therapeutic agent for the treatment of PD. Natural plants and their derivatives are being utilized as an effective medication for treatment of various disorders since years as they possess many important biological activities such as anti-inflammatory, anti-oxidative, anti-cholinesterase. *Desmodium gangeticum*, *Aloe vera*, *Ocimum sanctum*, *Moringa oliefera*, *Inonotus obliquus* plants are being utilized as natural drugs for treatment of PD but their mechanism of action is yet not very clear. In this study we are going to study the interactions of these plants active constituents with LRRK2 kinase protein with the help of protein ligand docking. Therefore, the present study is an attempt for better understanding of the mechanism of action of these plants active constituents as well as comparative study of their effects in the treatment of PD.

INTRODUCTION

Neurodegenerative diseases are nervous system disorders in which progressive degradation of spinal cord and brain nerve cells starts resulting in either functional loss (ataxia) or sensory dysfunction (dementia). It is affecting millions of people throughout the world. Neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), Epilepsy, Multiple sclerosis, Amyotrophic lateral sclerosis, Huntington's disease and many others are caused by many factors like genetic factor, free radical formation, mis-folding of protein, mitochondrial dysfunction and oxidative stress etc.

Parkinson's disease-

Parkinson's disease was firstly described as a syndrome by James Parkinson in 1817 in an essay on shaking palsy [1]. Later in 19th century Charcot gave credit to James Parkinson by naming it as Parkinson's disease (PD). It is a gradually progressing disorder of nervous system which causes involuntary shaking movements, tremors etc. According to a study, number of individuals dying from the disease is increasing gradually as 103,000 deaths were reported in year 2013 which was 44,000 in year 1990 [2].

Symptoms-

There are four main characterizing features of Parkinson's that is grouped as abbreviation TRAP where T stands for Tremors, R stands for rigidity of muscles, A for akinesia and P for postural inability. Akinesia which is also called as Bradykinesia is the most observable feature of PD. It refers to slowing down of movement and results in difficulties with planning and execution of movement, performing the simultaneous tasks [3]. Both motor and non-motor features are associated with PD symptoms. Motor features include tremors, freezing, postural instability, rigidity, slow muscular movement while non-motor features include autonomic dysfunction, cognitive and behavioral dysfunction, sleep disorders etc. [4].

Causes-

PD is also known as idiopathic Parkinsonism as in some patients the cause of the disease is not well known and understood. But in some cases, there might be genetic as well as environmental factors may be associated with development of disease. Exposure to pesticides, insecticides, heavy metals etc. are some environmental factors which are known for causing PD.

Though in most of the cases the cause of PD in patients is not based on genetic regions but in some cases mutations in some genes are known to cause PD. These genes are referred as *PARK* genes [6]. Till now, identification of 6 *PARK* genes has been done which encode-

1. α-synuclein (*PARK*1/4)
2. Parkin (*PARK*2)
3. PINK1 (PTEN-Induced Kinase 1—*PARK*6)
4. DJ-1 (*PARK*7)
5. LRRK2 (*PARK*8)
6. ATP13A2 (*PARK*9).

Mutations in both *LRRK2* and *α-synuclein* result in development of autosomal dominant inherited disease while autosomal recessive disease is caused by mutations in *DJ-1*, *ATP13A2*, *PINK1* and *parkin*. Accumulation of proteinaceous aggregates, called as Lewy Neurites (LN) or LewyBodies (LB) as well as degeneration of dopaminergic neurons is observed in the substantia nigra in affected individuals [7]. When neuropathological analysis of LB and LN was done in PD patients it was has confirmed that it mainly consists of α-synuclein positive aggregates [8]. Many studies show that the soluble oligomers which are formed in the presence of some inhibitory compounds may not be toxic to nerve cells and thus inhibition by such compounds may have therapeutic potential for PD resulted because of α synucleinopathies [9]. Similarly, some experimental studies suggest that mutation in Leucine-rich repeat kinase-2 (LRRK2) is one of the most common causes of Parkinson's disease. So identifying the inhibitors of LRRK2 kinase can provide a potential target for the treatment of Parkinson's disease [10].

Diagnosis-

Most specialists use the United Kingdom Parkinson’s Disease Society's Brain Bank Diagnostic criteria [11]. The NICE (National Institute for Health and Clinical Excellence) guideline advocate that individuals with suspected PD should be referred quickly to specialist expertise in medical diagnosis. They advocate that all patients with suspected PD ought to be reviewed frequently and if atypical options develop than diagnosis should be reviewed.

It is troublesome for specialists to differentiate tremor from PD in the cases where asymmetric postural and action tremor of limbs seem at rest. In such conditions, single

photon emission computed tomography (SPECT) is beneficial and is suggested by NICE [12].

A cocaine derivative is tagged with gamma ray emitting isotope and administered intravenously. This tagged isotope binds to pre-synaptic dopamine reuptake site in the striatum and can be visualized by a gamma camera. Uptake is reduced in the patients suffering with PD.

LRRK2 gene-

Leucine-rich repeat kinase 2 (LRRK2) is a kinase enzyme which is encoded by LRRK2 gene in human. In 2004, studies revealed the association of mutation in this gene with the PD [13, 14]. Later on various biochemical, genetic, cellular studies provided insight into mechanism involved in development and pathogenesis of PD due to mutation into LRRK2 gene. Numerous inhibitors of LRRK2 kinase are being identified, which can show protective behavior against LRRK2-induced degeneration of neurons. Thus LRRK2 kinase inhibition seems to be a potential new therapeutic agent for the treatment of PD [10].

Role of natural plants in the treatment of PD-

Natural products and their derivatives are being used as conventional therapy for thousands of years for treatment of many disorders. According to a report, 55-60 percentage of the total drugs are being represented by natural product derived drugs from 1981–2006 [15, 16].

(A) *Innonotus obliquus*-

Belonging to the family of basidiomycetes, *Inonotus obliquus* is a black parasitic fungus which is known for its biotechnological utility and its potential as a source of many pharmaceuticals [17]. Many experimental studies that variety of secondary metabolites such as triterpenoids , polyphenolic compounds, melanins, ergosterol peroxide, steroids etc. are produced by *inonotus* spp.[18-20]. These secondary metabolites are having many biological activities like hepatoprotective, anti-tumor, hypoglycemi, antioxidant etc. [21]. Extract of *Inonotus obliquus* consist of many compounds like inonotic acid, iso-hispidin, inotilone, hispidin, (E)-4-(3,4-dihydroxyphenyl)but-3en-2-one etc. [22]. Hence the biological activity of such compounds can be utilized in treatment of neurodegenerative disorders like Alzheimer's, Parkinson's etc. where anti-oxidant activity plays a major role [23].

(B) *Aloe barbadensis miller-*

Belonging to family of Asphodelaceae (Liliaceae)*, Aloe barbadensis miller* which is also known as Aloe vera is a pea green colored plant and is being used extensively for medicinal purposes since centuries. It can be grown in various regions of India like Gujrat, Tamilnadu Rajasthan and Andhra Pradesh and is cultivated in various countries like Haiti, South Africa, USA, Venenzuela [24]. It has thin, transparent and tasteless gel which is a rich source of various compounds like enzymes, vitamins, phenolic compounds, minerals, sugars, lignin, saponins, sterols, salicylic acid and amino acids. Its active constituents possess numerous medicinal properties like anti-inflammatory, laxative, analgesic, anti-oxidative, and anti-pyretic. It prevents free radical damage by stopping the production of reactive oxygen in biological membranes thus protecting cellular membranes from oxidative damage which is one of the main causes of neurodegenerative disorders [25, 26].

(C) *Ocimum sanctum-*

Ocimum sanctum, also known as holy basil or Tulsi, is considered as a sacred plant in Hindu culture for its various pharmacological uses. It is a small, aromatic annual herb which can grow up to 15-18 inches in length and belongs to family Labiatae. Leaves of Tulsi are rich in various essential oils constituting of compounds like linaool, eugenol, carvacrol, euginal, polyphenols etc. [27]. Many pharmacological effects for example anticancer, radioprotecive and antifertility are exhibited by this plant. The active constituents of the plant are known to exhibit cardio protective effects and observed to be anti-apoptotic and anti-oxidant. They can diminish oxidative stress which is one of the causative factors for neurodegenerative disorders like AD, PD [28, 29].

(D) *Moringa oleifera-*

Belonging to flowering plant family Moringaceae, *Moringa oleifera* which is also called as horseradish tree or drumstick tree, is an economically important, fast growing plant and is found in north western regions of India. It is cultivated and used in various other countries as vegetable, herbal medicines [30]. Every part of this plant is a rich source of various minerals, vitamins and is being extensively used for various disorders in the folk medicine of South Asia, to heal inflammation and infectious disorders along with hematological, cardiovascular, kidney, gastrointestinal and hepatic diseases [31]. Leaves act as a good source of natural antioxidants due to the presence of various types of antioxidant compounds such as

flavonoids, carotenoids, phenolic and ascorbic acid. Numerous studies report that these compounds not only exhibit antioxidant property but also show memory facilitating effect [32].

(E) *Desmodium gangeticum*-

It is one of the most important plants used in Ayurveda, folk medicine and belongs to Fabaceae family. It is also known as Shalparni and roots of this plant are one of the main materials used for ayurvedic preparations. It is useful in curing various diseases like asthma, typhoid, dysentery and piles. The roots of the plants are known to possess CNS depressant activities, analgesic, anti-bacterial, immunomodulatory, and anti-inflammatory [33, 34]. The presence of sterols, flavones, glycosphingolipid, trytamines, phospholipids and heptadecanol are reported to be responsible for antioxidant activity of the plant. Thus it can be considered as an important drug candidate for neurodegenerative disorder [35].

REVIEW OF LITERATURE

Gradual loss in function and structure of nerve cells of brain and spinal cord may result in various disorders which are known as neurodegenerative diseases. Millions of people in the world are suffering from various neurodegenerative diseases like Huntington's diseases (HD), Parkinson's (PD), amyotrophic lateral sclerosis (ALS), Alzheimer's (AD), epilepsy etc. There are many factors which are responsible for these diseases like aging, protein aggregation, neuronal death, mitochondrial dysfunction, oxidative stress, altered protein modifications, and environmental factors [36].Several late onset neurodegenerative disorders like Parkinson's disease, Alzheimer' disease is generally caused by depositions of various toxic proteins which vary from disease to disease. Therefore it is important to understand the process of accumulation of these toxic proteins [37].

3.1. Parkinson's disease-

Parkinson's disease is known to be second most common neurodegenerative disease affecting mainly elder population. James Parkinson first described PD in 1817, though various cases of the disease can be found in historical documents. He reported about the 6 cases of the person suffering from Parkinson's disease earlier known as paralysis agitans. Later Jean-Martin studied the difference between Parkinson's disease and multiple sclerosis and various other diseases characterized by tremor, and gave the term Parkinson's disease [38].

Determination of the epidemiological figures related to PD is determined approximately as different studies vary according to criteria of diagnosis, methodology applied and population being studied. Prevalence rate of the disease vary according to age as rate of prevalence is approximately 1%–2% in the population of age group 60–65 year. According to a report of 2005, more than 4 million people are suffering from the PD throughout the world. Some reports also suggested that prevalence of PD depends on gender of the person as male-to-female ratio of about 3:2 is observed in most studies [39].

3.1.1 Symptoms-

The first step to identify the disease is to be well aware of the characteristic features of the disease which is considered as one of the main challenges in treatment of PD as symptoms and signs are often subtle. Four main characterizing features of Parkinson's are grouped as abbreviation TRAP. TRAP stands for the main four symptoms of PD which are Tremor, Rigidity, Akinesia and Postural instability. Tremors are observed in 70-100% of people who

are suffering with Parkinson's disease. Some patients suffer from slow vertical jaw or tongue tremor or postural tremors. Rigidity of muscles is observed in more than 90% of the people. Cogwheel rigidity which is a peculiar kind of rigidity in which jerks are felt by patient along with rigidity in muscles, is also observed in the patients with PD. 80-100% of patients with PD show symptoms of akinesia in which impairment of voluntary activity of muscles is observed. Postural instability is less observed in initial stages of PD but it may prevail with progression of disease [40].

Symptoms of PD are mainly classified into two categories-

1. Motor symptoms
2. Non motor symptoms

1. Motor symptoms-

Motor symptoms mainly include four features: rigidity, rest tremor, bradykinesia, rest tremor, rigidity, and postural and gait impairment. PD which is characterized on the basis of motor symptoms is called as *Parkinsonism*. Various other motor symptoms are decreased arm swing, difficulty while sitting and sleeping, dysphagia, dystonia and slow performance in daily activities [41].

2. Non motor symptoms-

While studying the symptoms of PD main emphasis is given to motor features as these can be observed easily as compared to non-motor which needs a medical expertise to recognize disease. However, since last few years there has been an increasing concern in studying the non-motor symptoms of PD. Some of the non-motor symptoms are hallucinations, illusions, anxiety, sleep disorders, constipation, restlessness, excessive sweating and urinary and sexual dysfunction [42].

3.1.2 Main causes of PD-

Formation of fibrillar aggregates known as Lewy bodies is one of the characteristic features of the PD. α-synuclein is considered to be one of the many identified molecules which constitute the LB fibrils [43]. Degeneration of pigmented neurons in the brainstem due to Lewy body is main pathology of PD. One can visualize the affected neurons of the substantia nigra pars compacta using microscope.

Till now 18 specific chromosomal regions, also known as chromosomal locus, have been identified and are named as PARK. These regions are numbered according to the sequential order of their recognition as PARK2, PARK3 etc. 6 genes have been identified which are

linked to these locus SNCA, LRRK2, PINK1, DJ-1, AT13A2, Parkin. Autosomal dominant forms of PD are associated mainly with the mutations in SNCA, LRRK2 gene while mutations in the Parkin, PINK1 and DJ-1 are responsible for the autosomal recessive form [44].

3.2 LRRK2-

Being a complex, multi-domain protein Leucine rich repeat kinase 2 (LRRK2) is considered as an important target for potential disease-modifying therapeutic methods for Parkinson's disease (PD). LRRK2 is phosphorylated in mammalian cells and brain hence treatment of cells with LRRK2 kinase activity inhibitors can stimulate LRRK2 dephosphorylation [45].

Several families suffering from PD were identified in which genetic inheritance was observed but they did not any of the previously known PD genetic mutations. This observation led to the discovery of LRRK2 PD. Prior to which, known Mendelian forms of PD were early-onset diseases, usually showing in the 30s, with autosomal recessive [PARK2 (parkin), PARK7 (DJ-1), PARK6 (PINK-1),] or dominant [PARK1 (α-synuclein)] patterns of inheritance. In comparison, several families in Europe, United States, and Asia were found to develop a dominantly inherited disease which was similar to PD, started in the 1950 -1960 and followed a course of the sporadic disease. In 2002, genome- wide linkage analysis of Japanese kindred discovered PARK8 locus on chromosome 12p11.2-q13.1. This development led to the elucidation of several other PARK8 families. Mutations were identified in LRRK2 in 2 families which were found to be the main reason behind PARK8 PD in 2004. In 2005, a missense mutation was observed in the activation loop of the LRRK2 kinase of 13 families of North American and European origin, which results in change in residue 2019 from glycine to serine ("G2019S"). In addition, mutation R1441H which has been identified in PD patients is located at the same codon where two confirmed mutations which are mutation R1441C and R1441G) are located [46].

In Asian population, a mutation in the WD40 domain, G2385R, has been observed as one of the factor for development of PD. The five initially described mutations (R1441C, R1441G, Y1699C, G2019S, and I2020T) are the primary ones to cause disease in large families, though many other mutations have been known to modify disease risk. Meanwhile LRRK2 mutations were identified in another five families, four out of which were from the Basque region of Spain and showed different missense mutation at codon 1441, resulting in change from arginine to glycine (herein"R1441G"). The fifth family belonged to English ancestry

and showed the same Y1699C mutation as seen in Family A. Furthermore, a missense mutation (isoleucine to threonine; "I2020T") in the activation loop of the kinase domain was identified in a Japanese family [47, 48].

LRRK2 belongs to ROCO protein family and is characterized by presence of a 200-250 amino acid Roc (Ras of Complex protein) domain, followed by a domain termed COR (C-terminal of Roc) consisting of 300-400 amino acid length. The Roc and COR domains are then followed by a kinase domain. About 40 members of the ROCO superfamily are found in a variety of species including, plants, mammals, dictyostelium, metazoa and prokaryotes. Sequence analytic studies of the protein shows that the protein consists of several domains which are, a leucine-rich repeat domain, a Roc domain followed by its COR domain, a C-terminal WD40 domain and a mitogen-activated protein kinase kinase kinase (MAPKKK) domain. Several pathogenic amino acid substitutions and many mutations which are known to be associated with disease are distributed throughout the functional domains of LRRK2, indicates that this protein may act as an upstream central integrator of various signaling pathway which are required for proper functioning of neurons. In addition, the presence of both protein interaction domains and enzymatic domains within LRRK2 indicates the role of protein as a scaffold for assembly of a multi-protein signaling complex [49].

As LRRK2 mutations are one of the main causes of Parkinson's disease, so inhibitors of LRRK2 kinase have been identified, that shows protective behavior in *in vitro* and *in vivo* models of LRRK2 induced degeneration of neurons. The studies showed that LRRK2 induced neurodegeneration *in vivo* is kinase dependent and that inhibition of LRRK2 kinase may prove to be a potential new neuroprotective paradigm for the treatment of Parkinson's disease [50].

3.3. Role of natural plants in treatment of PD

3.3.1 *Inonotus obliquus*-

Mushrooms are functional as well as nutritional food and also a source of physiologically beneficial medicines. In Russian traditional medicine, extracts of the mushroom *Inonotus obliquus* (Chaga) is used as an antitumor and diuretic [51]. Moreover Chaga has therapeutic effects, such as immunomodulatory, hepatoprotective and anti-inflammatory effects [52]. The compounds having an immunomodulatory effect improve the cognitive enhancing abilities

[53]. Various classes of secondary metabolites which are known to have anti-oxidant activity are also produced by medicinal mushroom.

Inonotus obliquus (Chaga in Russia, Kabanoanatake in Japan) is a parasitic fungus found on birch in the colder regions of Europe, Japan and Korea. In Russia, it is usually known as Chaga, it's a black shapeless form of fungus which after 10—15 years of parasitism on trunks, mainly of birch, has been used for medicinal preparations [54]. In recent times, reports have been published which shows health promoting functions of Chaga, such as, protection of DNA from oxidative stress, anti-inflammatory, anti-nociceptive and anti-tumor activities [55,56]. But no report has been published about the chemical structures of ingredients of Chaga except some terpenoids.

Moreover, it is known that oxidative stress is involved in several diseases [57-59] for example autoimmune diseases, neurodegenerative (AD, PD etc.), cancer, and hypertension. Hence many antioxidant ingredients from foods or other natural sources are being investigated for diseases protection and treatment [60]. For the same reason, antioxidant property of Chaga attracts attention. Although it was found that polyphenolic fraction of Chaga extract have antioxidant activity, the structures of the antioxidant principle were not clear [61].

Chaga is made up of two parts, black (mainly outside) and brown (mainly inside) that were previously known as Sclerotium (ST) and Fruiting body (FB), respectively but most of Chaga products in Japan were processed without distinguish them [62].Extraction of *Inonotus* spp., with ethanol and CHCl3: MeOH gives inonotic acid, inotilone, (E)-4-(3,4-dihydroxyphenyl)but-3en-2-one, hispidin and iso-hispidin, which were found to show anti-inflammatory activity [63].

3.3.2 *Aloe barbadensis miller*-

Aloe barbadensis miller which is also called as aloe vera, is documented for its numerous medicinal uses. It is also grown as a decorative plant throughout the world. Various products produced from various parts of the plant are used in the healing of various ailments.

It is a cactus-like perennial plant which grows well in dry and hot climatic conditions. Its stem is thick and short in size and grows up to 35-60 cm in height. The leaves of the plant are green colored, concave in shape of length approximately 30-60 cm with thickness of 1.8-2 cm and 10 cm broad. The center of the leaves consists of mucilaginous tissues which are rich of

gel like substance known as aloe vera sap. It has yellow colored flower and life span of 12 years [64].

Chemical analysis of *Aloe vera* reported to be constituted of about 75 nutrients and 200 active compounds such as sugar, vitamins, anthraquinones, minerals, saponins, enzymes, amino acids, lignin and salicylic acid [65]. Complex sugar molecules like acemannan are present in leaves gel and are known to have immunostimulating action. Outermost part of the skin contains anthraquinones which are known to be responsible for the plant's laxative properties [66-67].

Various scientific studies reported that aloe vera possess various pharmacological properties such as anti-fungal, anticancer, analgesic, antioxidant, antimicrobial, neuroprotective, hepatoprotective and wound healing [68]. Acemannan which is a derivative of mannan diminishes the symptoms of several neurological disorders. Aloe vera extract contains prostanoids which is a precursor of arachidonic acid and it is studied that some neurological problems are associated with variations in arachidonic pathway metabolism resulting in development of neurological disorder [69]. Rise in level of dopamines is also reported with use of aloe vera which can be helpful in treatment of PD. [66].

3.3.3 *Ocimum sanctum*-

Ocimum sanctum L. (also called as *Ocimum tenuiflorum*, Tulsi) is being used for its diverse medicinal properties since ancient times in Ayurveda. It is one of the holiest and most worshipped of the many medical herbs of the orient. It is considered to be an adaptogen, which helps to maintain different processes in the body and beneficial for adapting to stress [70]. Characterized by its strong scent and astringent taste, it is considered as a type of 'elixir of life' in Ayurveda and known to promote long life span. As an Ayurvedic remedies, it is used in treatment of various ailments such as common colds, headaches, various forms of poisoning, heart disease, stomach disorders, inflammation and malaria. Consumption of Tulsi is done in various forms such as herbal tea, dried power or fresh leaf which is also used as an insect repellent in storing grains [71]. It is an erect, highly branched sub-shrub with 30-60 cm in length. It has purplish flowers and green or purple leaves which have strong aroma and stem has hairy structures on it. Leaves are up to 5cm in length and have petiole and are ovate. Tulsi is distributed throughout the world tropics and is cultivated for religious and pharmacological purposes.

It contains various vitamins and minerals such as calcium, iron, several phytonutrients and vitamin C which are known to enhance digestion and absorption thus maintain good metabolism of body [72]. Chemical analysis of the extracts of plant reported the presence of two principal groups of compounds; first one is terpenoids and second is phenolic derivatives. About 25 terpenoid and fatty acid derivatives are reported to be active constituents present in the plant extract and reported to possess anti-cancerous, anti-microbial, anti-HIV and anti-inflammatory properties [73]. Various phenolic substances also reported in the extract from the leaves of O. sanctum such as eugenol, hydroxycinnamic acid derivatives, eugenol glycosides, benzoic acid derivatives and flavonoids and their glycosides. These compounds are known to exhibit antioxidant, anti-stress, anti-microbial, anthelmintic, anti-inflammatory and radio-protective activities [74]. Active constituents of the plants are known to reduce oxidative stress which is one of the main reasons behind PD for example linalool which is present in plant extract is known to stimulate dopamine concentration and hence various scientific studies suggest that the plant can be studied further for the anti-Parkinson's kind activity [75, 76].

3.3.4 *Moringa oliefera-*

Moringa oleifera also known as Drumstick tree is indigenous to Asia and widespread in many parts of Africa. It is one of the economically important species native to dry tropical areas in the Northwestern India, at the Southwestern foot of the Himalayas [77]. It is cultivated in various countries and can be grown in any tropical regions of the world which has temperature around 25–35 °C. Sandy or loamy soil with pH ranging from slightly acidic to slightly alkaline and a net rainfall of 250–3000 mm are optimum conditions for production of this plant [78]. High germination rates are achieved by the direct seeding method as seeds have the ability to germinate within 5–12 days after completion of seeding stage which can be implanted at a depth of 2 cm in the soil.

Almost all parts of this plant serve the purpose of providing nutrition and medicine and have been used for various disorders in the folk medicine of South Asia [79]. Its leaves are traditionally used as purgatives and in curing headaches, hemorrhoids, fevers, inflammation of nose and throat, eye and ear infections, bronchitis and. Leaves are also used as vegetables and can be cooked and consumed like spinach or for preparation of soups and salads[80]. They are also used to combat hypertension and cholesterol as well as it possesses anticancer, antitumor, anti-inflammatory, diuretic properties and analgesic activities were reported.

Presence of high amounts of polyphenols is known to contribute towards its antioxidant properties. Experimental studies of aqueous extract of leaf, fruit and seed reported the antioxidant properties, anti-quorum sensing (QS) potentials as well as the ability to prevent the oxidative DNA damage [81].

3.3.5 *Desmodium gangeticum*-

Desmodium gangeticum also called as Shalaparni is an is extensively utilized as traditional medicine in India and various other parts of sub-continent due to broad spectrum therapeutic potentiality over a long period of time. It is a sub-tropical perennial herb which grows in dry hill areas, usually in the basement of Western ghat region and Himalayan territory. It is an ascending shrub which can grow up to 2 to 4 feet in length. It has the numerous prostrate branches with angular and woody stem. Leaves are rounded shape and which are covered with various grey colored numerous thrones. Size of flowers is small and is purple or white colored. Seeds are pale yellow and small in size shaped as kidney. Flowering‑fruiting occurs usually during March to December [82]. Ayurvedic literatures describe its potentiality as regulator of nervous (Vata), venous (Pitta) and arterial (Kapha) systems which are essential to maintain good health. Experimental studies report that the ability of the plant extract and its active constituents for example desmodin, hordenine and gangetin as anti-amnesic, immunostimulative, anti-diabetic, anti-inflammatory, antioxidant, cardio-protective, and hepatoprotective drug. Roots and aerial parts of the plant contain some phenolic and other phytochemical compounds which are responsible for its anti-oxidant activity [83].

MATERIALS AND METHODOLOGY

4.1 Bioinformatics web servers and tools used-

4.1.1 Protein Data Bank

Protein Data Bank (PDB) (URL:www.rcsb.org/) is depository which is freely accessible to public and serves the purpose of storing structural data of large biomolecules which are obtained by experimental studies like X-ray crystallography and NMR spectroscopy.

4.1.2 Uniprot database

Uniprot is a protein database which is high quality, comprehensive and freely accessible and provides sequence and functional information about the protein. It is accessible at URL: www.uniprot.org/.

4.1.3 Chemspider database

Chemspider is a database which is a repository for storage of chemical structure of a large number of unique molecules, their physical and chemical properties and nomenclature. The 2D structure and IUPAC name and molecular properties of the selected ligands were retrieved from Chemspider database. It is accessible at URL: www.**chemspider**.com/.

4.1.4 CASTp Server

CASTp (Computed Atlas of Surface Topography of proteins) which is accessible at following URL: sts.bioe.uic.edu/**castp**/, is one of the tools which can be used for the functional site prediction. 3 D structure of the protein is used as input to predict the active site residue in a given protein molecule.

4.1.5 MEGA6

Mega (Molecular Evolutionary Genetics Analysis) is an integrated, freely accessible tool which can perform sequence alignment, can estimate molecular evolution rate, and can generate evolutionary trees.

4.1.6 Pymol

Pymol is freely accessible software which is used for visualization of a protein molecule. This software can further be used to edit protein structure by removing unnecessary ligands, heteroatoms, removal and addition of hydrogen which are attached to the protein molecule.

4.1.7 Chemsketch

Using chemsketch freeware, the 2 D structures (in '.mol' format) of the ligands were converted into 3 D structures with the help of 3 D optimization tool. Chemsketch is a freely accessible package which allows us to draw chemical structure

4.1.8 Autodock 4.2

It is a freely accessible docking tool which is utilized for studying the protein-ligand interaction. On the basis of various algorithms, binding energy of the docked complex can be calculated which is used to interpret the stability of the complex formed.

4.2 Methodology

4.2.1. Sequence retrieval

Uniprot (www.uniprot.org) was used to retrieve the fasta sequence of LRRK2 protein of Homo sapiens (human). Uniprot is a protein database which is high quality, comprehensive and freely accessible and provides sequence and functional information about the protein. It is formed by merging three different protein databases- (a) Swiss-Prot (developed by Swiss institute of bioinformatics and European bioinformatics institute), (b) TrEMBL (Translated European molecular biology laboratory), (c) PIR-PSD (Protein information resource- protein sequence database) [84].

4.2.2 Structure of LRRK2

The Research Collaboratory for Structural Bioinformatics (RCSB) is maintaining PDB since 1998 [85]. The three-dimensional structures of LRRK2 were obtained from the protein databank, PDB ID: 2zej.

4.2.3. Sequence analysis

The retrieved FASTA sequence for both the proteins was analyzed and studied by following the below given steps-

(A) Protein BLAST was done by using the retrieved FASTA sequence**.** BLAST (Basic Local Alignment Search Tool) is an algorithm which compares the primary biological sequence information. It compares query sequence with a large database of sequences and identifies sequences which match with query sequence above specific threshold value [86].

(B) Sequences from different organisms were selected which were identical to the query sequences and their FASTA sequences were downloaded and saved in the '.fasta' format.

(C) Using ClustalW option of Mega 6.0 tool, the multiple sequence alignment of the sequences was performed. Mega (Molecular Evolutionary Genetics Analysis) is an integrated, freely accessible tool which can perform sequence alignment, can estimate molecular evolution rate, and can generate evolutionary trees. ClustalW is a widely used program for the multiple sequence alignment of divergent species [87].

(D) Pairwise distance of the multiple aligned sequences was calculated using Mega 6.0 tool

4.2.4. Structure analysis-

The sequence was analyzed for studying the properties of function, domain, properties and its active site residues. Uniprot provides a lot of information about its function and role in human body.

(A) Ramachandran plot analysis-

The Ramachandran plot displays the conformation angles (Phi and Psi) of the polypeptide chain of a protein molecule. For Ramachandran plot analysis, we used the Ramachandran plot on web 2.0 tool [88]. In this tool, PDB id of the protein is chosen as input and various options for representing the conformation angles in various regions, for indicating the conformation angles in secondary structural elements and regions according to the user specified Phi and Psi values in the plot are available. The updated version of the tool is accessible at the following URL: http://dicsoft1.physics.iisc.ernet.in/rp/.

(B) Domain prediction -

From Uniprot cross reference section, we got the PFam reference of our sequence. Pfam is a protein family database which contains their annotations and multiple sequence alignment generated by HMM (Hidden Markov Models). It provides useful information about the protein families and domains [89].

(C) Physico-chemical properties-

Protparam tool of Expasy (Expert protein analysis system) which is a proteomic server was utilized (http://web.expasy.org/protparam/) for computation of physic-chemical properties of the protein. Protparam is a tool which is used to calculate the various physical and chemical properties of the given protein sequence. It calculates various parameters like molecular weight, stability index, GRAVY (Grand average of hydropathy), aliphatic index, extinction coefficient and theoretical pI [90].By inputting, the sequence of proteins in Protparam tool, various physical and chemical parameters of the protein was calculated.

4.2.5 Active site prediction

Active site of the enzyme is that part of enzyme where substrate molecule can bind with the enzyme and is involved in catalytic reaction. Amino acid residues which are present in active site of the enzyme are known as active site residues. Knowledge of active site residues helps in ligand –protein docking, there are various tools available for the prediction of active site residues. CASTp is one of the tools which can be used for the functional site prediction [91]. 3 D structure of the protein is used as input for the prediction of active site residue.

4.2.6. Ligand preparation-

The list of ligand molecules were searched through various literature papers. Chemspider is a chemical database which contains structure of various molecules [92]. 2D structure and IUPAC name of the selected ligands were retrieved from Chemspider database. Using chemsketch freeware, the 2 D structures (in '.mol' format) of the ligands were converted into 3 D structures with the help of 3 D optimization tool. Chemsketch is a freely accessible package which allows us to draw chemical structure, calculate various properties like molecular weight, pI and viewing 3 D structures by importing the 2 D structures [93].

4.2.7 Evaluation of molecular properties of ligands

Molecular properties of all the ligands were retrieved from Chemspider database. On the basis of Lipinski's rule of five, which helps to differentiate between drug like and not drug like molecule, few ligands were selected.

4.2.8. ADMET prediction

The SMILE (Simplified molecular-input line-entry system) format of the ligands was taken as input for prediction of various ADMET (Absorption, Distribution, Metabolism, Excretion, Toxicity) properties using online server, Pkcsm Pharmacokinetic server. It is a freely accessible novel server (http://structure.bioc.cam.ac.uk/pkcsm) which employs use of graph-based signatures to develop predictive models of central ADMET properties for selecting suitable drug candidate [94]. Molecular descriptors like Water solubility, carcinogenicity Skin Permeability, AMES toxicity, CNS permeability and side effects such as mutagenicity, and teratogenicity were determined.

4.2.9 Docking studies

(A) Preparation and loading of protein in AutoDock 4.2:

a. Protein file was selected by clicking the 'read molecule' option under 'File Tab'.
b. Then hydrogen molecules were added by selecting the 'add hydrogen' option in 'Edit tab'.
c. Similarly Kollman charges were added using 'Edit tab' and AD4 type was assigned to atoms
d. After editing our target protein, the protein molecule was saved in the '.pdbqt' format.

(B) Loading of ligand in AutoDock 4.2:

a. Ligand was loaded in '.pdb' format in the autodock tool by selecting 'input' option under 'ligand tab'.
b. The ligand was saved in '.pdbqt' format.

(C) Setting up the grid:

a. The saved protein file in '.pdbqt' format was chosen by selecting the 'macromolecule' option in the 'grid tab'
b. For setting map Types, saved ligand file with '.pdbqt' extension was selected.
c. Then grid box was set up by entering the parameters in such a way that it can cover all the active site residues of the target protein and then setting grid box file was saved with '.gpf' extension.

(D) Setting the Docking parameters:

a. For setting the docking parameters, protein file and ligand file with '.pdbqt' extension were selected by clicking on 'macromolecule' option under 'docking' tab.
b. Genetic algorithm was selected for performing the docking study and default settings

were chosen for docking parameters and autodock 4.1 parameter

(E) Performing the AutoDock Run:

a. All the files generated in the process were saved under same directory.

b. Then we run the command **"autogrid4.exe –p abdc.gpf –l abdc.glg"** followed by the **"autodock4.exe –p abcd.dpf –l abcd.dlg"** command using DOS prompt and obtained the result file in '.dlg' format.

(F) Analysis of docking file:

a. The docked file obtained in the '.dlg' format was opened by clicking on 'docking' option under the 'analyze' tab.

b. This file contains only ligand so protein molecule was attached by selecting the 'macromolecule' option in 'analyze' tab.

c. Various conformations can be analyzed and saved by playing the conformations according to the rank. Rank number one is given to the conformation having minimum binding energy and interactions can be visualized under 'analyze' tab.

RESULTS AND DISCUSSION

5.1 Structure of Leucine-rich repeat serine/threonine-protein kinase 2(LRRK2)

The sequence of LRRK2 (Leucine-rich Repeat serine/threonine protein Kinase 2) was obtained from Uniprot having Uniprot ID **Q5S007**. The three-dimensional structure of LRRK2 was obtained from the protein databank, PDB ID: **2ZEJ** with a resolution of 2.00Å (as shown in figure 1) having both chains A, B and two ligands. This structure was viewed using Pymol viewer.

Figure 1 – Structure of LRRK2 (PDB ID: 2ZEJ) visualized using Pymol viewer

LRRK2 (EC: 2.7.11.1) is a 2527 amino acid protein which is encode by LRRK2 gene. It is also known as Dardarin protein. It provide helps in regulation of neuronal process morphology in the central nervous system (CNS) and have an important role in synaptic vesicle trafficking. It may be helpful in the phosphorylation of proteins related to Parkinson disease. In our work, LRRK2 protein with PDB ID 2ZEJ having chain A, B comprising of 184 amino acid is studied.

5.2 Sequence analysis-

5.2.1 Blast result of the target protein-

Using BLASTP, we found that LRRK2 protein with Uniprot ID- QS50007.2 shows 100 % query coverage and 90-97% identity results in the following organisms as listed in Table 1.

Table 1 – Blast result of LRRK2 using protein blast showing identity (%) and Query coverage (%) in different organisms

S.No	Accession ID	Organism	Max score	Total score	Query coverage(%)	E-Value	Identity(%)
1	Q5S007.2	Homo sapiens	5202	5202	100	0	100
2	NP_001162390.1	Papio anubis	5039	5039	100	0	97
3	ELR59783.1	Bos mutus	4757	4757	100	0	91
4	DAA29845.1	Bos taurus	4754	4754	100	0	91
5	NP_001106908.1	Sus scrofa	4710	4710	100	0	91
6	EHB08723.1	Heterocephalus glaber	4643	4643	99	0	90
7	ELK07161.1	Pteropus alecto	4614	4614	100	0	90

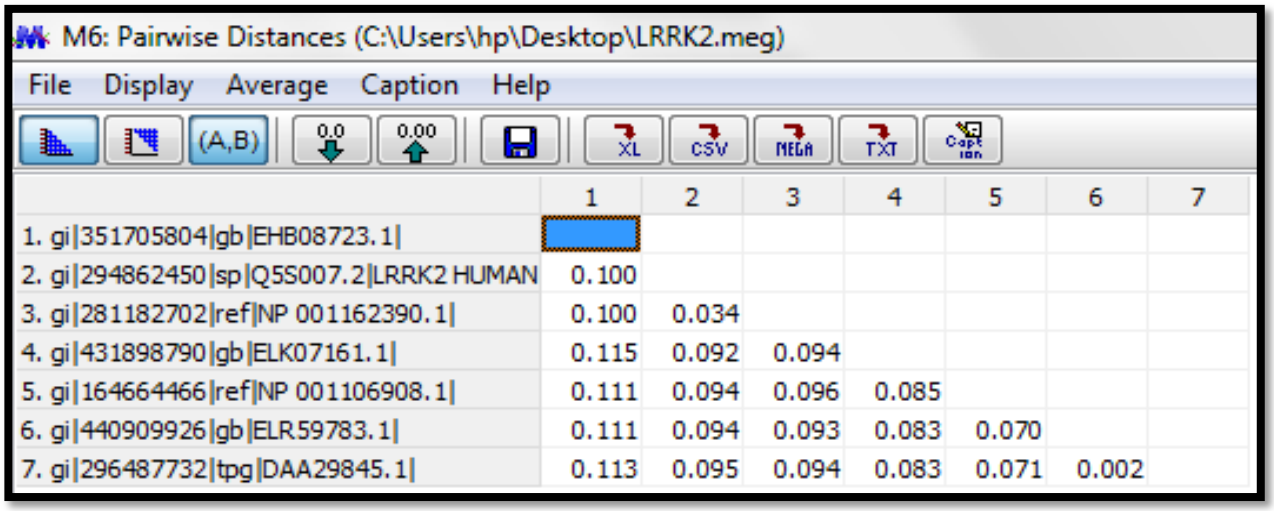
Papio Anubis (olive baboon) with accession id NP_001162390.1 is the most identical to *Homo sapiens* as it is having maximum identity as well as total score as compared to other organisms.

5.2.2 Multiple sequence alignment of the target protein-

The fasta sequences of LRRK2 protein of different organism were aligned using ClustalW tool in Mega 6.0 to find out the extent of similarity among the sequences of different organisms. The result of the alignment is shown in figure 2. Analyzing the result of multiple sequence alignment, we can find out that maximum number of sequences are showing complete alignment with a very few gaps mainly in the sequence of *Pteropus alecto* having accession id ELK07161.1.

Figure 2 - Multiple sequence alignment of LRRK2 protein in different organism using Mega 6.0 tool

5.2.3 Pairwise distance calculation of the target protein in different organisms-



	1	2	3	4	5	6	7
1. gi|351705804|gb|EHB08723.1|							
2. gi|294862450|sp|Q5S007.2|LRRK2_HUMAN	0.100						
3. gi|281182702|ref|NP_001162390.1|	0.100	0.034					
4. gi|431898790|gb|ELK07161.1|	0.115	0.092	0.094				
5. gi|164664466|ref|NP_001106908.1|	0.111	0.094	0.096	0.085			
6. gi|440909926|gb|ELR59783.1|	0.111	0.094	0.093	0.083	0.070		
7. gi|296487732|tpg|DAA29845.1|	0.113	0.095	0.094	0.083	0.071	0.002	

Figure 3 – Pairwise distance of LRRK2 protein in different organisms using MEGA 6.0

Similarly pairwise distance calculation using MEGA6.0 was performed to find out the relationship between the various organisms for LRRK2 protein. The figure 3 shows that the distance between the organisms *Heterocephalus glaber* (Accession ID EHB08723.1) and *Pteropus alecto* (Accession ID ELK07161.1) is maximum. Distance is lowest between the organisms *Bos Taurus* (Accession ID DAA29845.1) and *Bos mutus* (Accession ID ELR59783.1). It means that organisms *Heterocephalus glaber* and *Pteropus alecto* are most distinctly related and organisms *Bos Taurus* and Bos *mutus* are most closely related.

5.2.4 Phylogenetic study of the target protein-

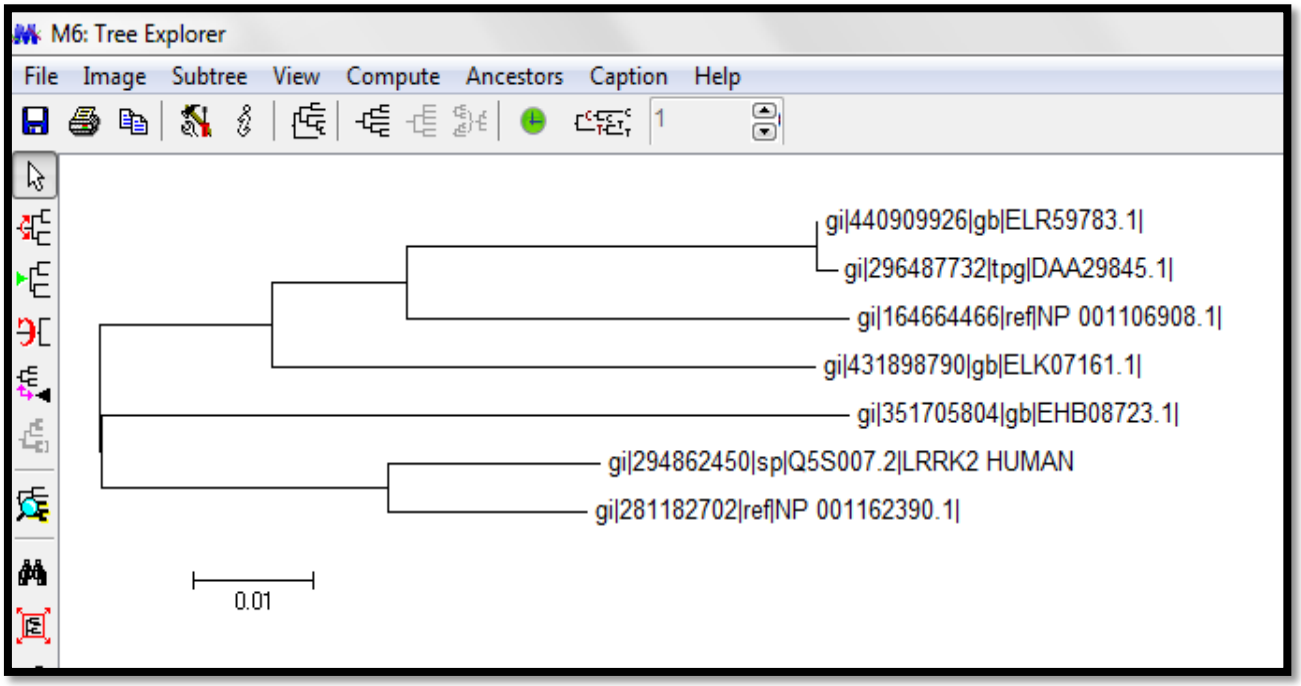


Figure 4–Phylogenetic tree analysis of LRRK2 protein in different organisms using Maximum likelihood tree using MEGA 6.0 tool

Phylogenetic analysis was done for the LRRK2 protein present in different organisms by constructing Maximum likelihood tree using MEGA6.0 tool. We observed from the tree that *Bos taurus* and *Bos mutus* are most closely related *and Heterocephalus glaber* and *Pteropus alecto* are distinctly related organisms.

5.3. Structure analysis of the target protein-

5.3.1 Ramachandran plot analysis of the protein-

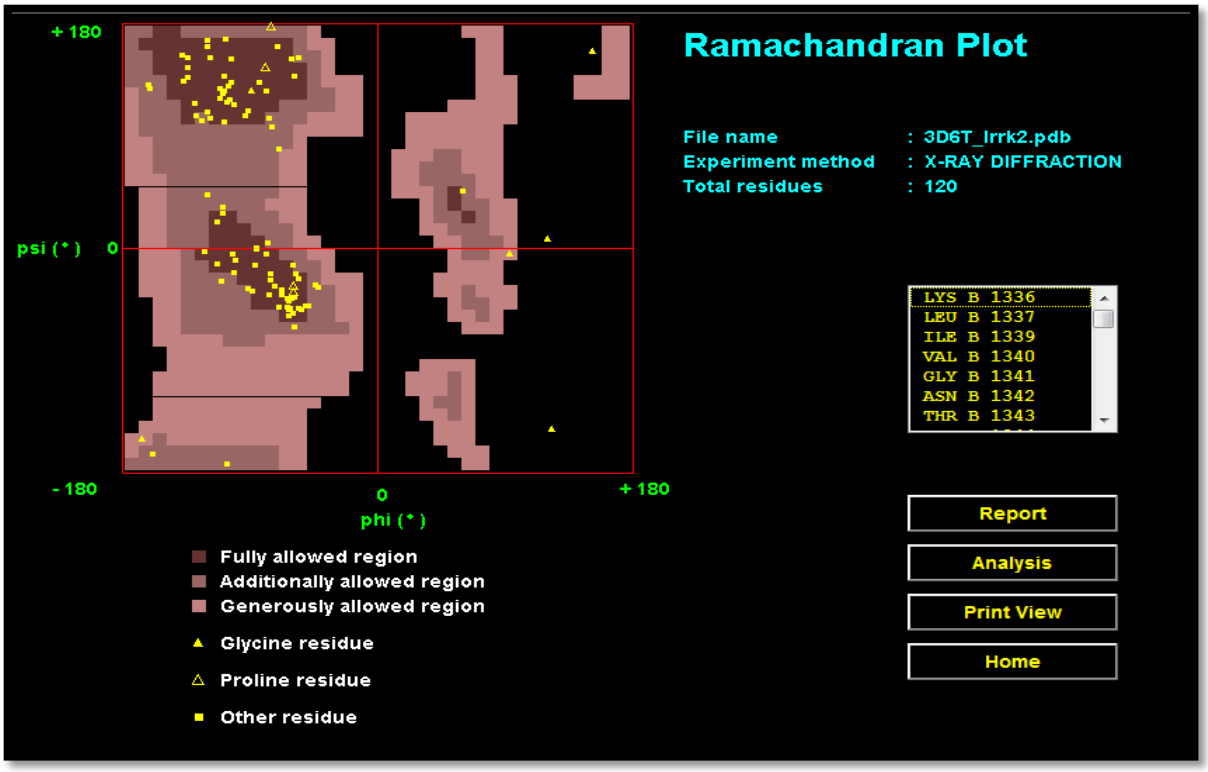


Figure 5 - Ramachandran plot of LRRK2 protein using Ramchandran Plot 2.0

In order to check that LRRK2 protein is a suitable model for docking, we referred the structural validity of LRRK2 Ramachandaran Plot 2.0 analysis web tool. The Ramachandran plot analysis for the LRRK2 protein structure (Figure 5) shows that LRRK2 is a good choice which is of excellent quality to use as a target for docking studies with the ligands. The residues percentage in most favored regions was 68.75% in LRRK2 indicating the target protein structure is of good quality with only 3.13% of residues in disallowed regions

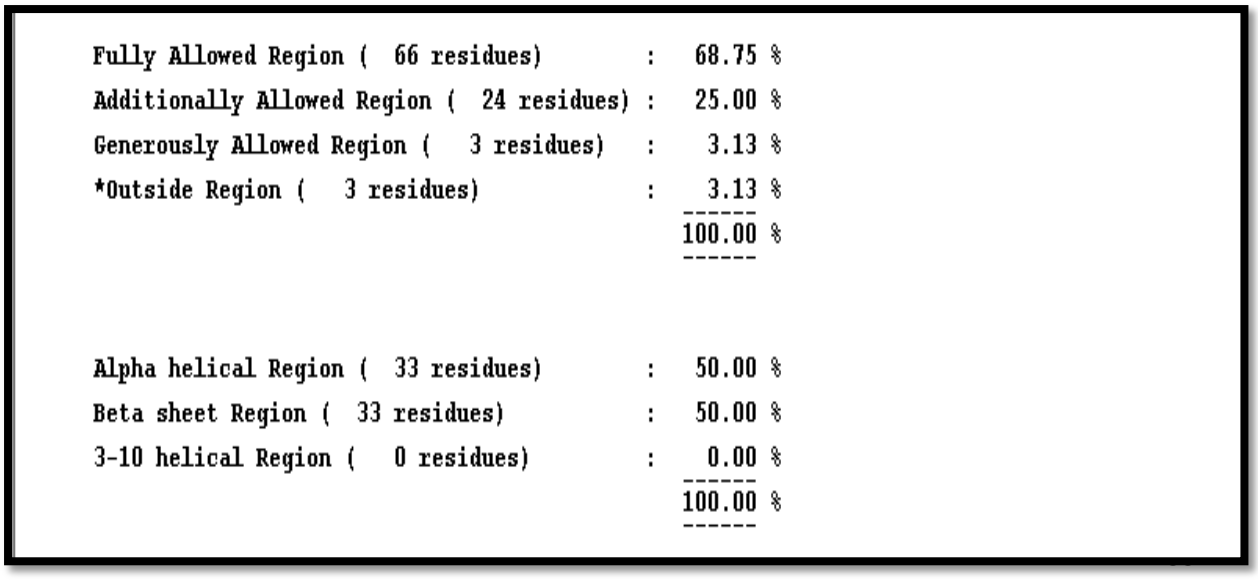
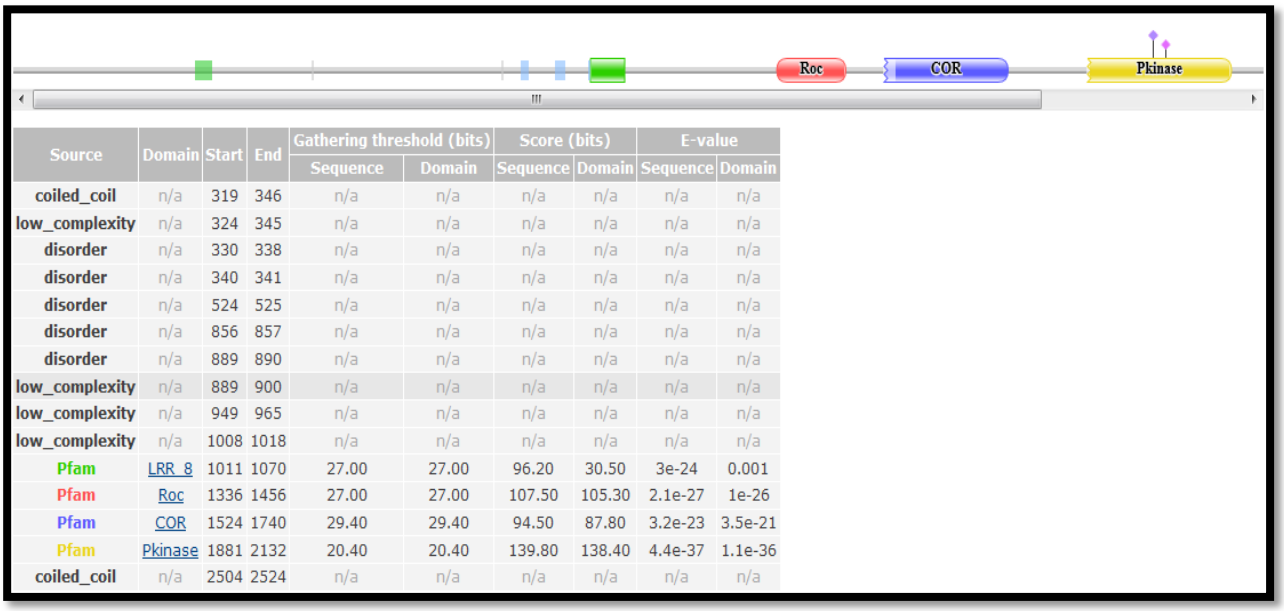


Figure 6– Percentage of Allowed and disallowed region in LRRK2 protein

5.3.2 Domain prediction of the target protein-



Source	Domain	Start	End	Gathering threshold (bits)		Score (bits)		E-value	
				Sequence	Domain	Sequence	Domain	Sequence	Domain
coiled_coil	n/a	319	346	n/a	n/a	n/a	n/a	n/a	n/a
low_complexity	n/a	324	345	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	330	338	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	340	341	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	524	525	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	856	857	n/a	n/a	n/a	n/a	n/a	n/a
disorder	n/a	889	890	n/a	n/a	n/a	n/a	n/a	n/a
low_complexity	n/a	889	900	n/a	n/a	n/a	n/a	n/a	n/a
low_complexity	n/a	949	965	n/a	n/a	n/a	n/a	n/a	n/a
low_complexity	n/a	1008	1018	n/a	n/a	n/a	n/a	n/a	n/a
Pfam	LRR_8	1011	1070	27.00	27.00	96.20	30.50	3e-24	0.001
Pfam	Roc	1336	1456	27.00	27.00	107.50	105.30	2.1e-27	1e-26
Pfam	COR	1524	1740	29.40	29.40	94.50	87.80	3.2e-23	3.5e-21
Pfam	Pkinase	1881	2132	20.40	20.40	139.80	138.40	4.4e-37	1.1e-36
coiled_coil	n/a	2504	2524	n/a	n/a	n/a	n/a	n/a	n/a

Figure 7– Domain prediction of LRRK2 protein using PFam

Domain of LRRK2 protein was predicted by using Pfam server. LRR_8, Roc, COR, Pkinase domains are present in the protein which starts from 1011 and ends at 2132 (as shown in figure 7). Gathering threshold, Score and E value of the domains as well as sequence is shown in figure 7.

5.3.3 Physico-chemical properties

With the help of PROTPARAM tool of EXPASY, the physico-chemical properties of LRRK2 were studied (as shown in table 2). The molecular weight of LRRK2 protein was found to be 28.61 kDa. pI is the pH at which net charge on protein becomes zero and it becomes stable. It was find out that the pI of LRRK2 is 6.35 which reveal that it is slightly acidic in nature as its pI < 7.0.

Instability index of a protein tells us about the stability of protein in a test tube. Protein molecule with instability index value< 40 is predicted as stable while value >40 predicts that the protein may be unstable .So it was find out that LRRK2 may be unstable as it was having 44.53.

Aliphatic index of LRRK2 is 103.02 which indicate that it may be stable for a wide range of temperature variation. GRAVY (grand average of hydropathy value) is sum of hydopathy

values of all amino acids divided by total number of residues in the sequence. GRAVY index of LRRK2 is -0.114 which indicates that it can show better interaction with water

Table 2 - Physico-chemical properties of LRRK2 protein

Length	2527 aa		
Sequence type	Protein		
Organism	Homo spaiens		
Weight	28.61 kDa		
Isoelectric point	6.35		
Instability index	44.53		
Gravy	-0.114		
Aliphatic index	103.02		
Half life			
N Terminal AA	Half-life mammals	Half life yeast	Half -life E.coli
Methionine	30 hours	>20 hours	>10 hours
Extinction coefficient			
Conditions	Extiction coefficient at 280 nm	Extinction coefficient at 280nm 0.1%(=1g/L)	
Non reduced cysteines	227460	0.795	
Reduced cysteines	223460	0.781	
Atomic composition			
Atom	Count		
Hydrogen	20461		
Carbon	12771		
Nitrogen	3435		
Oxygen	3730		
Sulphur	134		
Count of charged residues			
Charge	Count		
Positively charged	281		
Negatively charged	307		
Amino acid distribution			
Amino acid	Count	Frequency(%)	
Alanine (A)	127	5	
Arginine (R)	105	4.2	
Asparagine (N)	128	5.1	
Aspartic acid(D)	123	4.9	
Cysteine	64	2.5	
Glutamine (Q)	105	4.2	
Glutamic acid (E)	184	7.3	
Glycine (G)	119	4.7	
Histamine(H)	79	3.1	
Isoleucine (I)	160	6.3	
Leucine (L)	356	14.1	
Lysine(K)	176	7	
Methionine (M)	70	2.8	
Phenylalanine(F)	96	3.8	
Proline(P)	94	3.7	
Serine(S)	205	6.1	
Threonine (T)	96	3.8	
Tryptophan (W)	26	1	
Tyrosine (Y)	54	2.1	
Valine(V)	160	6.3	

5.3.4 Active site prediction-

Using CASTp server, we find out that 44 pockets are present in the LRRK2 protein. Out of the 44 pockets, pocket no 44 is having maximum surface are as well as volume (as shown in table 3), so we further analyzed this pocket for predicting active site residue

Table 3- Pockets predicted in LRRK2 protein using CASTp

S.No	Pocket No.	Area[Å²]	Volume[Å³]
1	44	787.6	968.6
2	43	699	949.2
3	42	355.3	369.8
4	41	303.9	331.8
5	39	139.1	162.5
6	40	138.6	139.5
7	38	106.5	129
8	34	104.4	119.5
9	37	99.3	77.3
10	36	88.2	93.5

On further analysis of pocket 44, we find out that various amino acids are present in the pocket as shown in table 4.

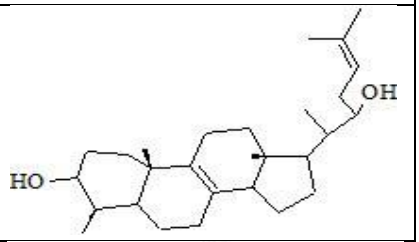
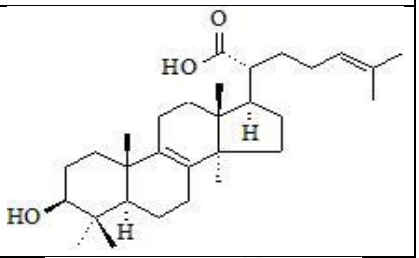
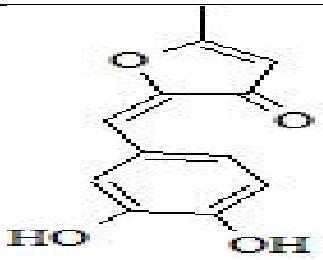
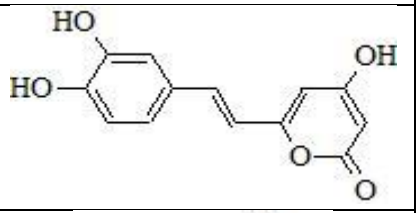
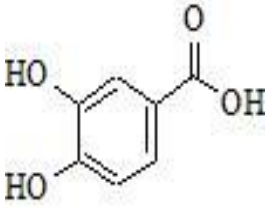
Table 4-Amino acid residue present in Pocket 44 predicted using CASTp server

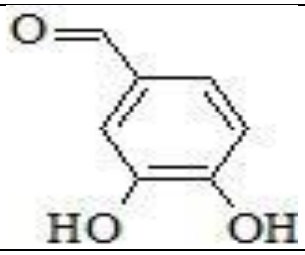
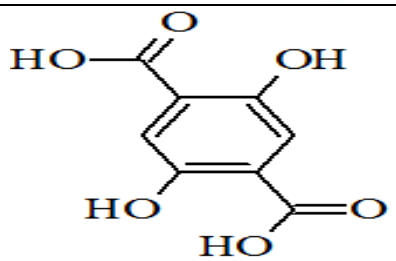
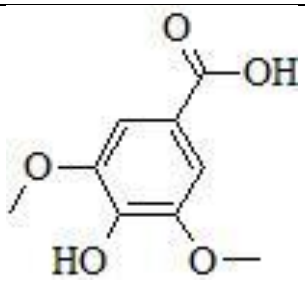
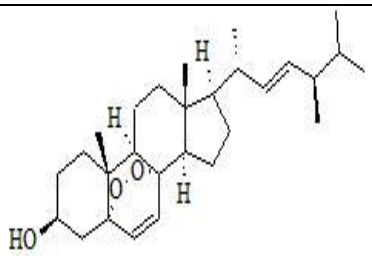
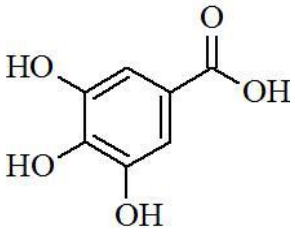
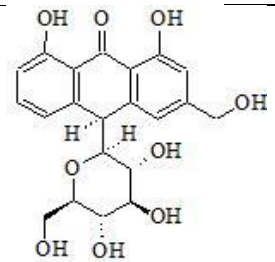
S.No.	Residue No.	Residue name(chain name)
1	1336	Lysine(B)
2	1337	Leucine(B)
3	1369	Valine(A)
4	1370	Glycine(A)
5	1371	Isoleucine(A)
6	1373	Valine(B)
7	1374	Lysine(B)
8	1375	Aspartic acid(B)
9	1388	Leucine(B)
10	1389	Valine(B)
11	1390	Leucine(B)
12	1391	Aspargine(B)

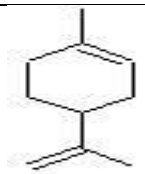
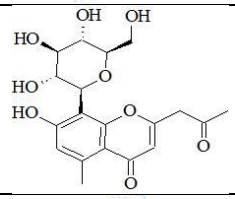
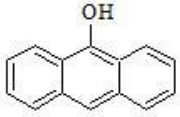
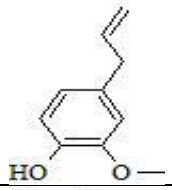
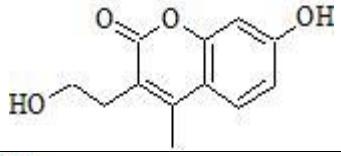
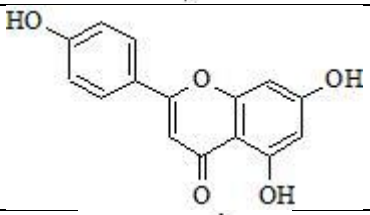
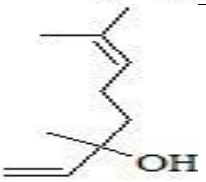
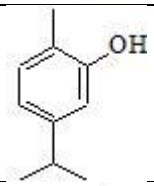
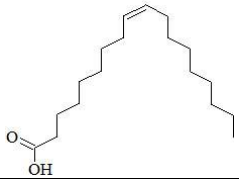
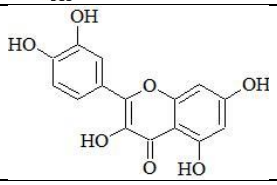
5.4. Ligand preparation-

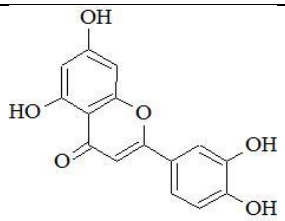
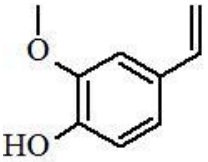
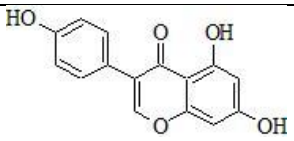
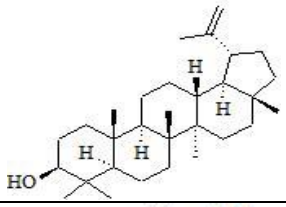
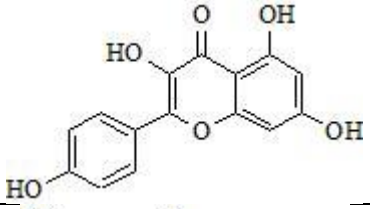
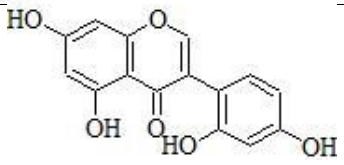
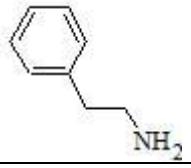
From Chemspider database, the 2-D structure and IUPAC name of 30 ligand molecules were retrieved. The 2-D structure of the compounds along with their Chemspider ID is shown in Table 5.

Table 5-List of ligands along with their 2-D structure and IUPAC name

Compound name	Chemspider ID	IUPAC name	2-D structure
Inotodiol	23282401	(3β,22R) Lanosta-8,24-diene-3,22-diol	
Trametenolic acid	10193870	(3β)-3-Hydroxylanosta-8,24-dien-21-oic acid	
Inotilone	22547096	(2E)-2-(3,4-Dihydroxybenzylidene)-5-methyl-3(2H)-furanone	
Hispidin	13975015	6-[(E)-2-(3,4-dihydroxyphenyl)ethenyl]-4-hydroxypyran-2-one	
Protocatechuic acid	71	3,4-Dihydroxybenzoic acid	

Protocatechuic aldehyde	8438	3,4-Dihydroxybenzaldehyde	
2,5-Dihydroxyterephthalic acid	62347	2,5-Dihydroxyterephthalic acid	
Syringic acid	10289	4-Hydroxy-3,5-dimethoxybenzoic acid	
Ergosterol peroxide	4508532	(1S,2R,5R,6R,9R,10R,13S,15S)-5-[(2R,3E,5R)-5,6-Dimethyl-3-hepten-2-yl]-6,10-dimethyl-16,17-dioxapentacyclo[13.2.2.$0^{1,9}$.$0^{2,6}$.$0^{10,15}$] nonadec-18-en-13-ol	
Gallic acid	361	3,4,5-Trihydroxybenzoic acid	
Aloin	24534069	(1S)-1,5-Anhydro-1-[(9S)-4,5-dihydroxy-2-(hydroxymethyl)-10-oxo-9,10-dihydro-9-anthracenyl]-D-glucitol	
Squalene	553635	(6E,10E,14E,18E)-2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene	

Limonene	20939	4-Isopropenyl-1-methylcyclohexene	
Aloesin	140797	(1S)-1,5-Anhydro-1-[7-hydroxy-5-methyl-4-oxo-2-(2-oxopropyl)-4H-chromen-8-yl]-D-glucitol	
9-anthrol	10278	9-anthrol	
Eugenol	13876103	4-Allyl-2-methoxyphenol	
Ocimarin	4450710	7-Hydroxy-3-(2-hydroxyethyl)-4-methyl-2H-chromen-2-one	
Apigenin	4444100	5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one	
Linalool	13849981	3,7-Dimethyl-1,6-octadien-3-ol	
Carvacrol	21105867	5-Isopropyl-2-methylphenol	
Oleic acid	393217	(9Z)-9-Octadecenoic acid	
Quercetin	4444051	2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one	

Luteolin	4444102	2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one	
p-Vinylguaiacol	325	2-Methoxy-4-vinylphenol	
Pentacosane	11900	Pentacosane	
Genistein	4444448	4H-1-Benzopyran-4-one, 5,7-dihydroxy-3-(4-hydroxyphenyl)	
Lupeol	228079	(3β)-Lup-20(29)-en-3-ol	
Kaempferol	4444395	3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one	
2-hydroxygenistein	4445299	3-(2,4-Dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one	
Phenethylamine	13856352	2-Phenylethanamine	

5.5 Evaluation of molecular properties of ligands-

The various molecular properties of ligand molecule was retrieved using Chemspider database in order to study the drug like properties such as molecular weight, H-bond donor, H-bond acceptor, log P and molecular formula of the ligand molecules. For a ligand to act like drug following criteria should be followed by that ligand-

(A)Molecular mass should be less than 500 Da.

(B)High lipophilicity which is indicated by log P should be less than5.

(C)Number of hydrogen bond donor should be less than5.

(D)Number of hydrogen bond acceptor should be less than 10.

According to the above criteria, limonene, phenethylamine, p-vinylguaiacol, eugenol, linalool, carvacrol, protocatechuic acid and protocatechuic aldehyde ligand molecules are exhibiting drug like behaviour.

Table 6-Molecular properties of ligands retrieved from Chemspider database

S.NO	**Ligand**	**Molecular formula**	**Molecular weight (Da)**	**H-bond acceptor**	**H-bond donor**	**logP**
1	Inotodiol	$C_{30}H_{50}O_2$	442.717	2	2	9
2	Trametenolic acid	$C_{30}H_{48}O_3$	456.7	3	2	8.96
3	Inotilone	$C_{12}H_{10}O_4$	218.205	4	2	1.42
4	Hispidin	$C_{13}H_{10}O_5$	246.215	5	3	0.57
5	Protocatechuic acid	$C_7H_6O_4$	154.12	4	3	1.16
6	Protocatechuic aldehyde	$C_7H_6O_3$	138.12	3	2	1.14
7	2,5-Dihydroxyterephthalic acid	$C_8H_6O_6$	198.13	6	4	2.91
8	Syringic acid	$C_9H_{10}O_5$	198.173	6	4	2.91
9	Ergosterol peroxide	$C_{28}H_{44}O_3$	428.64	3	1	7.68
10	Gallic acid	$C_7H_6O_5$	170.12	5	4	0.91

11	Aloin	$C_{21}H_{22}O_9$	418.394	9	7	1.86
12	Squalene	$C_{30}H_{50}$	410.718	0	0	13.09
13	Limonene	$C_{10}H_{16}$	136.234	0	0	4.45
14	Aloesin	$C_{19}H_{22}O_9$	394.373	9	5	0.64
15	9-anthrol	$C_{14}H_{10}O$	194.229	1	1	3.94
16	Eugenol	$C_{10}H_{12}O_2$	164.201	2	1	2.2
17	Ocimarin	$C_{12}H_{12}O_4$	220.221	4	2	2.03
18	Apigenin	$C_{15}H_{10}O_5$	270.237	5	3	2.1
19	Linalool	$C_{10}H_{18}O$	154.229	1	1	3.28
20	Carvacrol	$C_{10}H_{14}O$	150.218	1	1	3.28
21	Oleic acid	$C_{18}H_{34}O_2$	282.461	2	1	7.7
22	Quercetin	$C_{15}H_{10}O_7$	302.236	7	5	2.08
23	Luteolin	$C_{15}H_{10}O_6$	286.236	6	4	2.4
24	p-Vinylguaiacol	$C_9H_{10}O_2$	150.174	2	1	1.93
25	Pentacosane	$C_{25}H_{52}$	352.68	0	0	14.04
26	Genistein	$C_{15}H_{10}O_5$	270.237	5	3	2.96

27	Lupeol	$C_{30}H_{50}O$	426.717	1	1	10.98
28	Kaempferol	$C_{15}H_{10}O_6$	286.236	6	4	2.05
29	2-hydroxygenistein	$C_{15}H_{10}O_6$	286.236	6	4	2.99
30	Phenethylamine	$C_8H_{11}N$	121.18	1	2	1.46

5.6 ADMET prediction-

ADMET prediction helps us to understand the behavior of drug in our body. Various descriptors like Blood brain barrier (BBB), Human intestinal absorption (HIA), $CaCO_2$ permeability, CYP2D6 inhibitor, CYP3A4 inhibitor, AMES toxicity and hepatotoxicity were utilized in our study to examine the ADMET property of our ligands For a drug to pass the blood brain barrier, value of BBB should lie between -1.0 to 0.3. Ligand molecules should have CaCO2 permeability value greater than 0.90. Any ligand molecule having HIA (%) value less than 30% are known to have poor absorption. A ligand molecule should not inhibit CYP2D6 and CYP3A4 enzymes as well as it should not cause any toxicity.

Table 7- ADMET properties of ligands calculated using pkCSM tool

S.NO	**Ligand**	**BBB**	**HIA (%)**	**$CaC0_2$ permeability (log P_{app} in 10^{-6}cm/s)**	**CYP2D6 inhibitor**	**CYP3A4 inhibitor**	**AMES toxicity**	**Hepatotoxicity**
1	Inotodiol	0.377	91.269	1.255	NO	NO	NO	NO
2	Trametenolic acid	0.154	90.468	1.218	NO	NO	NO	YES
3	Inotilone	-0.038	93.886	0.93	NO	NO	YES	NO
4	Hispidin	-0.791	90.696	1.01	NO	NO	YES	NO
5	Protocatechuic acid	-0.752	63.432	0.147	NO	NO	NO	NO

6	Protocatechuic aldehyde	-0.28	74.867	1.186	NO	NO	NO	NO
7	2,5-Dihydroxyterephthalic acid	-1.139	30.874	-.507	NO	NO	YES	NO
8	Syringic acid	-0.374	76.013	0.277	NO	NO	YES	NO
9	Ergosterol peroxide	0.333	95.085	1.241	NO	NO	NO	YES
10	Gallic acid	-0.93	50.311	-0.467	NO	NO	NO	NO
11	Aloin	-1.065	49.874	-0.311	NO	NO	YES	NO
12	Squalene	1.04	90.913	1.351	NO	NO	NO	NO
13	Limonene	0.557	98.048	1.248	NO	NO	NO	NO
14	Aloesin	-1.187	56.214	-0.336	NO	NO	YES	YES
15	9-anthrol	0.31	95.316	1.388	NO	NO	YES	NO
16	Eugenol	0.348	96.594	1.48	NO	NO	NO	NO
17	Ocimarin	-0.032	95.139	0.249	NO	NO	YES	NO
18	Apigenin	-0.708	90.14	1.062	NO	NO	YES	NO
19	Linalool	0.502	95.91	1.356	NO	NO	NO	NO
20	Carvacrol	0.355	95.56	1.379	NO	NO	NO	NO
21	Oleic acid	0.024	91.219	1.492	NO	NO	NO	NO

22	Quercetin	-1.065	73.104	0.076	NO	NO	YES	NO
23	Luteolin	-0.886	79.391	0.27	NO	NO	YES	NO
24	p-Vinylguaiacol	0.285	96.318	1.474	NO	NO	NO	NO
25	Pentacosane	1.239	89.788	1.291	NO	NO	NO	NO
26	Genistein	-0.688	90.14	1.07	NO	NO	YES	NO
27	Lupeol	0.252	93.588	1.37	NO	NO	NO	NO
28	Kaempferol	-.0886	79.391	0.27	NO	NO	YES	NO
29	2-hydroxygenistein	-0.867	79.391	0.281	NO	NO	YES	NO
30	Phenethylamine	-0.029	84.905	1.209	NO	NO	NO	NO

On the basis of the above mentioned criteria, out of the 30 ligand molecules only 13 ligand molecule are exhibiting good ADMET properties which are squalene, limonene, lupeol, phenethylamine, oleic acid, p-vinylguaiacol, pentacosane, eugenol,linalool, carvacrol, inotodiol, protocatechuic acid, protocatechuic aldehyde.

5.7. Docking

On the basis of ADMET prediction and molecular properties evaluation, only 8 ligand molecules were considered for further protein ligand docking study.

5.7.1 Docking study of target protein with selected ligand molecules-

(A) Docking study of LRRK2 with Linalool-

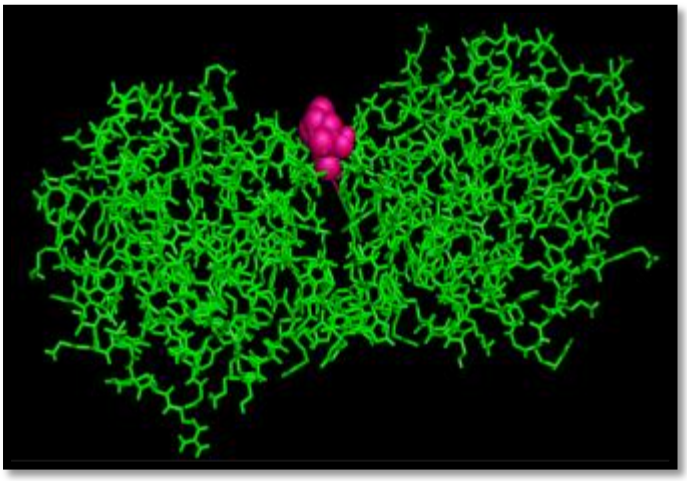


Figure 8 (A) - Docked complex of LRRK2 with Linalool

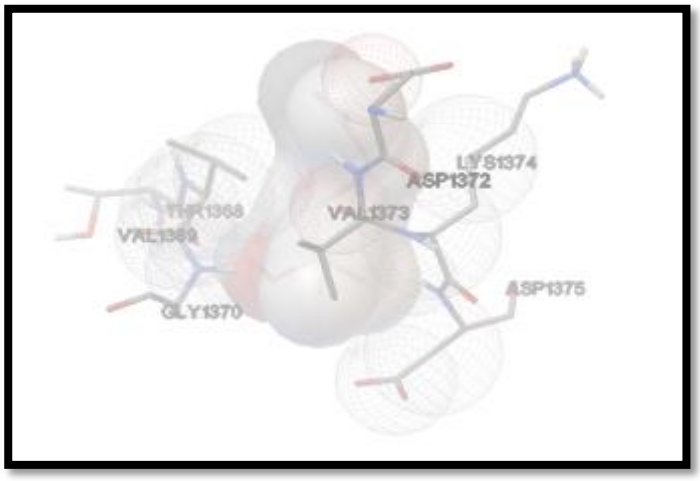


Figure 8 (B) - 3-D interaction of Linalool with LRRK2

Above figure 8(A) is showing the docked complex between target protein LRRK2 and ligand Linalool where pink colored spheres are indicating linalool molecule. This ligand is interacting with THR 1368, VAL 1369, VAL 1373, ASP 1372, ASP 1375, LYS 1374, GLY 1370 amino acid residues of the target protein (as shown in figure 8 (B)).

(B) Docked complex of LRRK2 with Eugenol

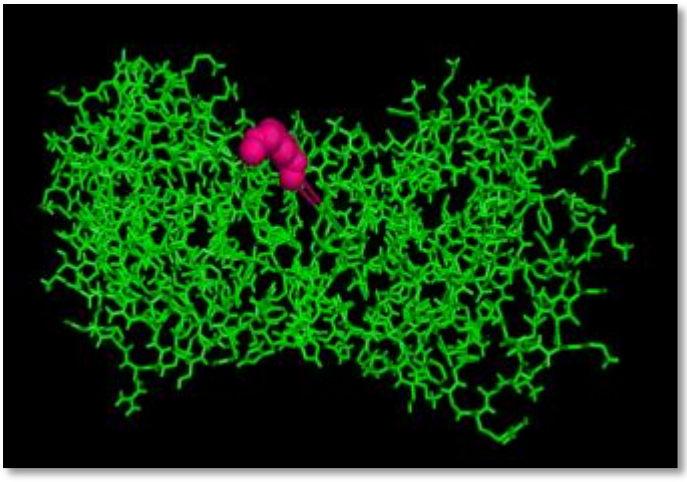


Figure 9 (A) - Docked complex of LRRK2 with Eugenol

Figure 9 (B) - 3-D interaction of Eugenol with LRRK2

Above figure 9(A) is showing the docked complex between target protein LRRK2 and ligand eugenol where pink colored spheres are indicating eugenol molecule. This ligand is interacting with PRO 1377, VAL 1369, VAL 1373, ASP 1375, LYS 1374, GLY 1370 amino acid residues of the target protein (as shown in figure 9 (B)).

(C) Docked complex of LRRK2 with Carvacrol

Figure 10(A) is showing the docked complex between target protein LRRK2 and ligand carvacrol where pink colored spheres are indicating carvacrol molecule. This ligand is interacting with MET 1335, LYS 1336, SER 1403, PHE 1406, GLU 1399, TYR 1402 amino acid residues of the target protein (as shown in figure 10 (B)).

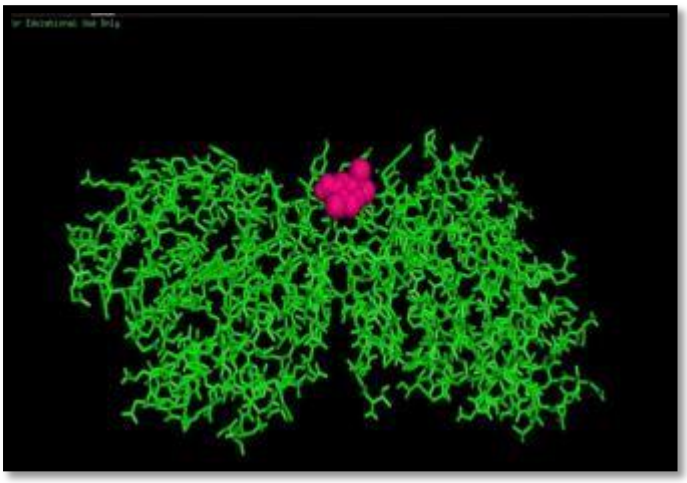


Figure 10 (A) - Docked complex of LRRK2 with Carvacrol

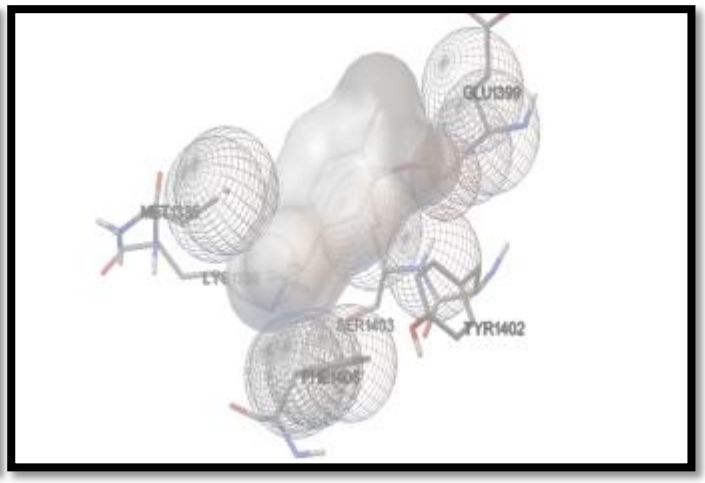


Figure 10 (B) - 3-D interaction of Carvacrol with LRRK2

(D) Docked complex of LRRK2 with p-vinylguaiacol

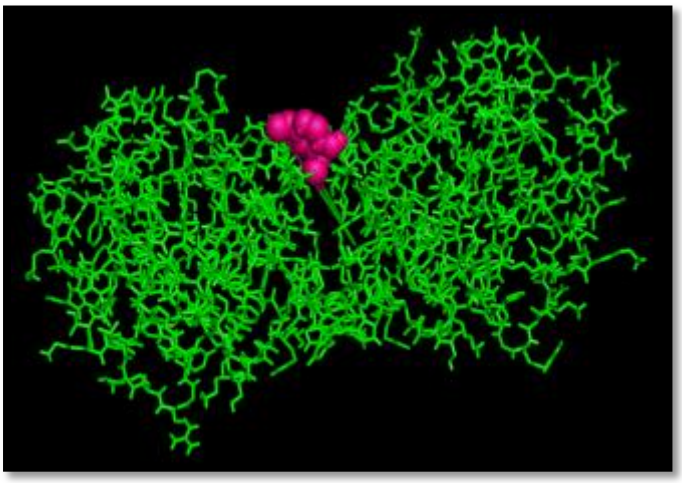


Figure 11 (A) - Docked complex of LRRK2 with p-vinylguaiacol

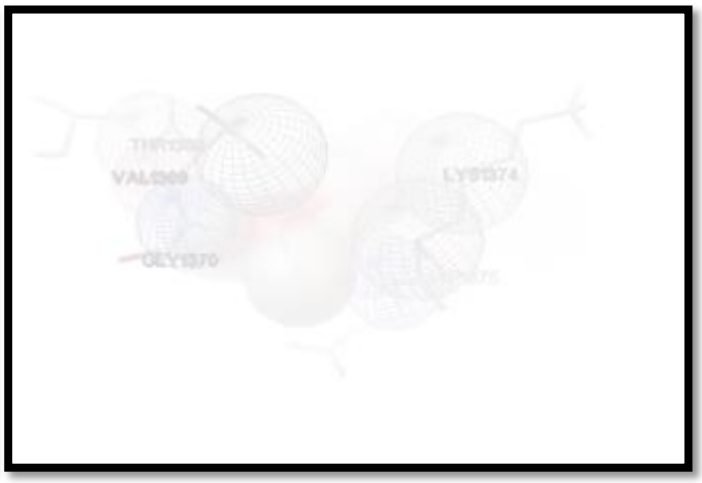


Figure 11 (B) - 3-D interaction of p-vinylguaiacol with LRRK2

Above figure 11(A) is showing the docked complex between target protein LRRK2 and ligand p-vinylguaiacol where pink colored spheres are indicating p-vinylguaiacol molecule. This ligand is interacting with THR 1368, VAL 1369, ASP 1375, LYS 1374, GLY 1370 amino acid residues of the target protein (as shown in figure 11 (B)).

(E) Docked complex of LRRK2 with Limonene

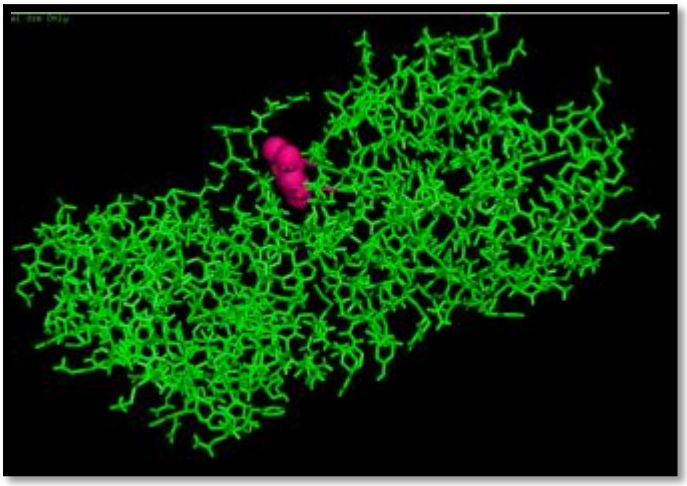


Figure 12 (A) - Docked complex of LRRK2 with Limonene

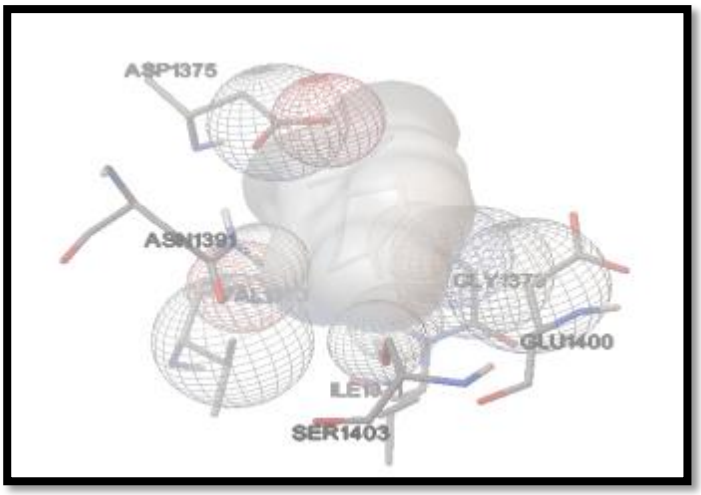


Figure 12 (B) - 3-D interaction of Limonene with LRRK2

Above figure 12(A) is showing the docked complex between target protein LRRK2 and ligand Limonene where pink colored spheres are indicating limonene molecule. This ligand is interacting with GLU 1400, ASN 1391, VAL 1373, SER 1403, ASP 1375, ILE 1371, GLY 1375 amino acid residues of the target protein (as shown in figure 12 (B)).

(F) Docked complex of LRRK2 with Phenethylamine

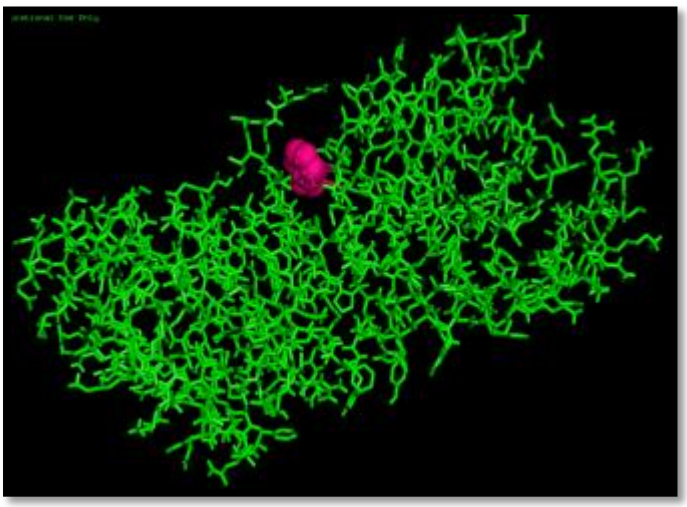


Figure 13 (A) - Docked complex of LRRK2 with Phenethylamine

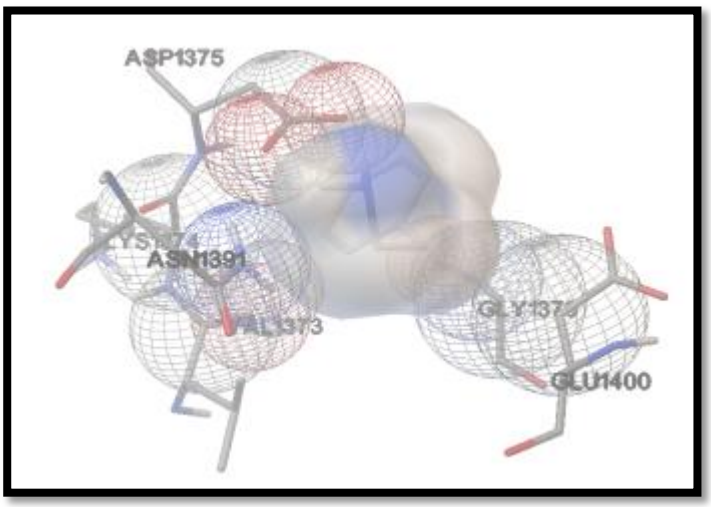


Figure 13 (B) - 3-D interaction of Phenethylamine with LRRK2

Above figure 19(A) is showing the docked complex between target protein LRRK2 and ligand phenethylmaine where pink colored spheres are indicating phenethylamine molecule. This ligand is interacting with ASP 1375, LYS 1374, ASN 1391, VAL 1373, GLU 1400 amino acid residues of the target protein (as shown in figure 19 (B)).

(G) Docked complex of LRRK2 with Protocatechuic aldehyde

Following figure 20(A) is showing the docked complex between target protein LRRK2 and ligand protocatechuic aldehyde where pink colored spheres are indicating protocatechuic aldehyde molecule. This ligand is interacting with ASP 1372, ILE 1371, VAL 1373, VAL 1369 amino acid residues of the target protein (as shown in figure 20 (B)).

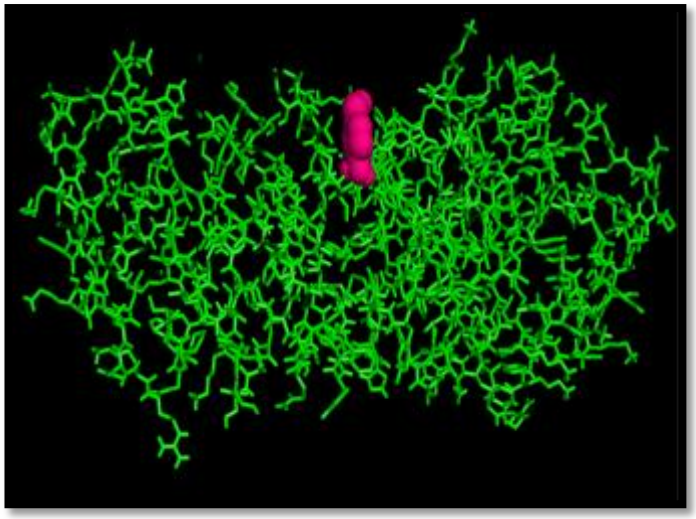


Figure 14 (A) - Docked complex of LRRK2 with Protocatechuic aldehyde

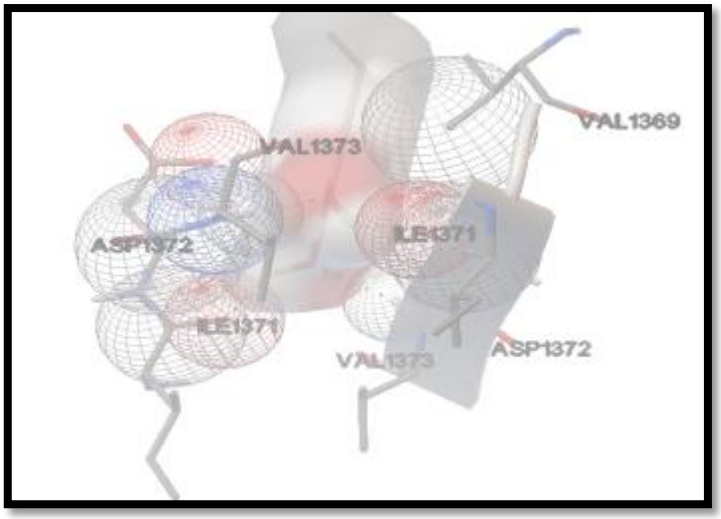


Figure 14 (B) - 3-D interaction of Protocatechuic aldehyde with LRRK2

(H) Docked complex of LRRK2 with Protocatechuic acid

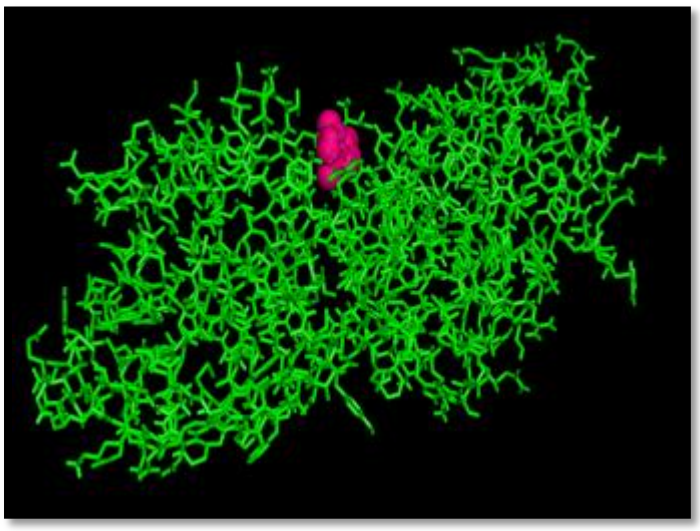


Figure 15 (A) - Docked complex of LRRK2 with Protocatechuic acid

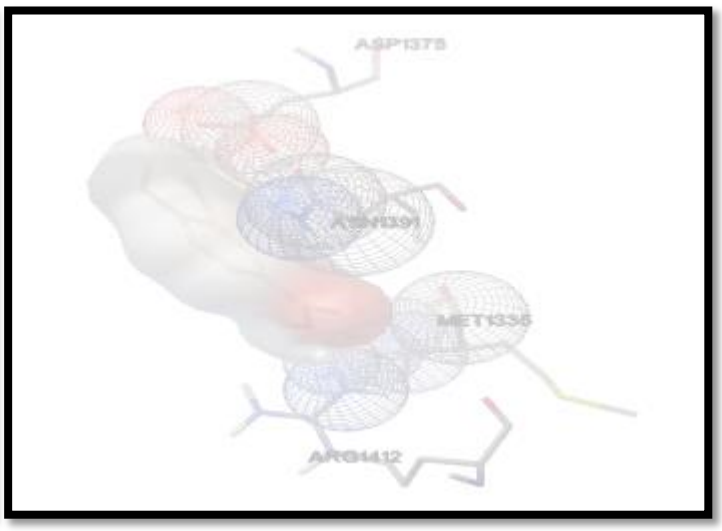


Figure 15 (B) - 3-D interaction of Protocatechuic acid with LRRK2

In the above figure 21(A) is showing the docked complex between target protein LRRK2 and ligand protocatechuic acid where pink colored spheres are indicating protocatechuic acid molecule. This ligand is interacting with ASP1375, ASN 1391, MET1335, ARG 1412 amino acid residues of the target protein (as shown in figure 21 (B)).

5.7.2 Docking energies-

Table 8- Docking energies of selected biomolecules calculated using Autodock 4.2

S.No	Ligand	RMSD (Aº)	Free binding energy(kcal/mol)	Final intermolecular energy(kcal/mol)	Final internal energy(kcal/mol)	Torsional free energy(kcal/mol)	Inhibition constant (mM)
1	Linalool	76.506	-1.98	-3.77	-0.35	1.79	35.28
2	Eugenol	79.131	-2.73	-3.93	-0.49	1.19	9.91
3	Carvacrol	57.042	-2.89	-3.48	-0.09	0.6	7.64
4	p-vinylguaiacol	78.451	-2.91	-3.8	-0.21	0.89	7.37
5	Limonene	61.443	-2.96	-3.26	-.11	0.3	61.443
6	Phenethylamine	61.87	-2.98	-3.88	-0.09	0.89	3.8
7	Protocatechuic aldehyde	70.062	-3.3	-4.2	-0.21	0.89	3.8
8	Protocatechuic acid	76.207	-4.54	-5.73	-0.4	1.19	0.471

In the above table no. 7, energies of the docked complex of target protein with ligand molecule is represented according to descending value of free binding energy. Ligand molecule having lowest free energy is the most stable docked complex and thus the best candidate for drug. Hence, Protocatechuic acid ligand molecule, which is an active

constituent of *Innonotus obliquus* plant, is the best drug candidate out of the selected ligand molecules.

CONCLUSION

Parkinson's disease (PD) is one of the most common neurodegenerative disease affecting millions of individuals worldwide. Although there are various treatments available for reducing the symptoms of PD but no one appears to show a promising role in prevention or even slowing the progression of the disease and also cause some side effects. In the present study of Parkinson's disease we have utilized the natural plant extracts as a drug candidate for treatment of Parkinson's disease. Extract of these plants are known to stimulate the nerve growth factor production, which are helpful in rapid development of healthy neurons and better mitochondrial function. Main active constituents of these herbs includes triterpenes, sterols, polysaccharides which are responsible for antioxidant and anti-inflammatory properties of the extracts make these good candidates for prevention of neurodegenerative diseases. 30 compounds which are active constituents of the plants were selected as ligand. Out of theses 30, 13 compounds were selected on the basis of their ADMET prediction. Only 8 compounds exhibited drug like properties when we studied their various molecular properties. We performed docking studies of these 8 compounds with our target protein and it was observed that protocatechuic acid is showing minimum binding energy. Thus all these eight ligand molecules, with protocatechuic acid having maximum potential, can be examined further by lab experiments for developing drug against PD.

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