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Exploring medicinal plants for Alzheimer's Disease: Qualitative Analysis and In-silico studies

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Submitted by:

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CANDIDATE'S DECLARATION

I Ramsha Usman, Roll No. 2K21/IBT/07 student of M.Tech (Industrial Biotechnology), hereby declare that the project Dissertation title "**Exploring medicinal plants for Alzheimer's Disease: Qualitative Analysis and In-silico studies**" which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

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CERTIFICATE

I hereby certify that the Project Dissertation titled "**Exploring medicinal plants for Alzheimer's Disease: Qualitative Analysis and In-silico studies**" which is submitted by Ramsha Usman, Roll No. 2K21/IBT/07, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is a record of the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

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ABSTRACT

The main purpose of the recent study was to use both qualitative and quantitative screening techniques to determine whether the leaf extracts of the two medicinal plants *Hibiscus rosa-sinensis* and *Nerium oleander* contained any phytochemicals. During the qualitative analysis, the phytochemical components, including carbohydrates, triterpenoids, terpinoides, glycosides, lignins, alkaloids, phenolic compounds, flavonoids, saponins, tannins, quinones, and anthroquinones, were screened in the plant extracts using accepted techniques. For 8 phytochemical tests, the plant's aqueous extract, *Hibiscus rosa-sinensis*, produced favourable findings. For ten experiments, the *Nerium oleander* extract produced favourable results. The use of standard qualitative tests are still frequently employed for the initial screening of the phytochemicals of plants, despite the fact that there are many advanced methods for determining phytochemicals. The pharmaceutical industry values plants for their extensive structural variety and diverse spectrum of pharmacological effects. A range of ailments have been treated with medicinal plants due to their possible pharmacological qualities. Antioxidant, antineoplastic, analgesic, anti-inflammatory and further activities are a few of these characteristics. The phytoconstituents of a medicinal plant, either individually or collectively, determine its therapeutic value. Alzheimer's disease, which is one of the most common form of dementia, is one of the greatest unmet medical needs. Due to poor pharmacokinetic qualities and safety concerns, there are fewer new compounds entering the market. The current work uses in silico experiments to demonstrate the molecular interactions of currently approved antipsychotic medications with the many protein targets linked to AD.

The key goal of the research work is to screen natural chemicals utilising computational methods for BuChE inhibition. Using the 3D structure of BuChE, docking-based virtual screening was conducted a to look for possible AD inhibitors. SwissDock was used to conduct docking studies between ligands and enzymes and to analyse the Lipinski Rule of Five in this work. It was hypothesised that various phytochemical substances will inhibit BuChE, respectively. When compared to Thioflavin T (-7.90 kcal/mol), one of the most well-known inhibitors of Butyrylcholinesterase (BChE), and many other inhibitors including decamethonium, propidium, huprine, and ethopropazine, the docking results showed that riboflavin and oleagenin have a prominent and promising inhibitory potential against BuChE. In contrast to the target molecule BuChE, these chosen phytochemical compounds demonstrated superior interactions, making them intriguing research subjects for follow-up investigations into potential Alzheimer's disease therapies.

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LIST OF SYMBOLS, ABBREVIATIONS

List of Abbreviations	
AD	Alzheimer's disease
SMILES	Simplified molecular input line entry system
ADME	Absorption, Distribution, Metabolism, and Excretion
RCSB	Research Collaboratory for Structural Bioinformatics
PDB	Protein Data Bank
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
Leu	Leucine
Val	Valine
SOD	Superoxide Dismutase
MDA	Malondialdehyde
NFTs	Neurofibrillary tangles
ChE	Cholinesterase
AChE	Acetylcholinesterase
BuChE/ BChE	Butyrylcholinesterase
ACh	Acetylcholine
BuCh	Butyrylcholine
APP	Amyloid-beta precursor protein
PAS	Peripheral anionic site
CAS	Catalytic active site
BACE 1	Beta-site APP cleaving enzyme 1
MAO A	Monoamine oxidase A
NMDA	N-methyl-D-aspartate
KOH	Potassium hydroxide
H2SO4	Sulphuric acid
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
NaCl	Sodium chloride
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity

CHAPTER 1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Phytochemicals can be defined as the substances that are produced by plants naturally. They influence how plants look, taste, and smell. Additionally, they are a component of a plant's built-in defence system against disease. These phytochemicals are becoming more and more well-known today because of their many medical uses. Asthma, arthritis, cancer, and other disorders are all prevented in part by phytochemicals [1]. These phytochemicals don't have any negative effects, unlike pharmaceuticals. The phytochemicals are additionally referred to as "man-friendly medicines" because they treat illnesses without endangering people.

This study's goal was to assess the therapeutic potential and phytochemical composition of dried leaf extracts from two important medicinal plants, *Hibiscus rosa-sinensis* and *Nerium oleander*. The plants are categorised as therapeutic plants due to their capacity to synthesise chemical compounds that are essential in avoiding different diseases, like neurological diseases, cancer, diabetes, and others. *Hibiscus rosa-sinensis* L. is a perennial woody decorative shrub with plenty of flowers that is widely cultivated in tropical regions. It is advised to utilise *H. rosa-sinensis* as herbal option for treating numerous ailments because prior analysis has shown it to have bioactive qualities [2, 3]. The family Apocyanaceae and the genus *Nerium* both contain the species *Nerium oleander*. In warm, subtropical regions, it normally develops into a shrub or small tree. Due to its high phytochemical content, *N. oleander* demonstrates a wide range of biological and pharmacological effects. Different phytochemicals such as carbohydrates, alkaloids, flavonoids, steroids, cardiac glycosides, and tannins are all abundant in nerium leaves [3, 4]. The qualitative analysis of the phytochemical contents, including tannins, saponins, flavonoids, alkaloids, quinones, terpenoids, cardiac glycosides, and so forth was done using the distinguished test procedure reported in the literature. The collection, extraction, and qualitative analysis of phytochemicals are the key topics covered in this dissertation.

1.2 **MEDICINAL PLANTS CHOSEN FOR THE STUDY**

The importance of therapeutic plants since ancient times has been suggested by numerous research. Below are descriptions of two constituent medicinal plants that are chosen for phytochemical profiling, botanical characteristics and ethanomedicinal applications in the current research endeavour.

The plants chosen are:

Plant I: *Hibiscus rosa-sinensis* from Malvaceae family

Plant II: *Nerium oleander* Linn. from Apocynaceae family

1.2.1 ***Hibiscus rosa-sinesis***

Here below in the Table 1.2.1.1 the following data represents the botanical taxonomy description of Hibiscus *rosa-sinesis* [5].

Table 1.2.1.1**:** Botanical Taxonomy Description of *Hibiscus rosa-sinesis*

Botanical Name	*Hibiscus rosa-sinensis*
Common name(s)	China Rose, Jasud, Shoe-Flower
Kingdom	Plantae
Sub-Kingdom	Tracheobionta
Super division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-Class	Dilleniidae
Order	Malvales
Family	Malvaceae
Genus	*Hibiscus*
Species	*rosasinensis*
Habit	Perennial shrub
Root	Tap root system
Stem	Vertical, cylindric, woody, and branching in the air
Leaf	Simple, petiolate, stipulate, serrate, glabrous, Alternating with a single cyme, axillary, and with a multicostate reticulate venation at the apex
Flower	Floral components are linked, bracteate, bracteolate, bisexual, big, showy, pentamerous, dichlamydeous, actinomorphic, complete, and hypogynous
Epicalyx	The margin of the calyx has five to eight bracteoles. They are wild and lush

Calyx	Sepals 5 are oddly positioned at the posterior and are green with gamosepalous valvate aestivation
Corolla	Five polypetalous, multicoloured, base-connected petals that twist during aestivation
Androecium	Around the style, a staminal tube is created by the fusion of many monadelphous filament stamens. Red is the staminal tube. The filament is transversely joined to the monothecous, yellow, reniform, extrorse anthers that dehisce transversely
Gynoecium	Larger ovary, pentacarpellary, and syncarpous. Numerous ovules per locule in a pentalocular ovary on an axile placentation. The design is straightforward, long, and thin, and it fits into the staminal tube
Fruit	It is generally abortive
Floral Formula	Br Brl ⊕ ♂ K(5) C5 A(∞) G(5)

1.2.2 ***Nerium oleander*** **Linn**.

Here below in the Table 1.2.2.1 the following data represents the botanical taxonomy description of *Nerium oleander* Linn. [6].

Table 1.2.2.1**:** Botanical Taxonomy Description of *Nerium oleander* Linn.

Botanical NAME	*Nerium oleander* Linn
Common name(s)	The Oleander, Kanher, Lal Karen
Kingdom	Plantae
Sub-Kingdom	Tracheobionta
Super division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-Class	Asteridae
Order	Gentianales
Family	Apocynaceae Juss.
Genus	*Nerium L.*
Species	*N. oleander*
Habit	Shrubs
Root	Fibrous roots
Stem	Large glabrous woody shrub with upright stems
Leaf	The leaves feature a cuneate leaf base, an entire leaf border, and a pointed apex. They can be up to 30 cm long and 4 cm wide, and they can be arranged in whorls or oppositely

FLOWER	Flowers on the ends of each branch are fragrant, vivid crimson, and spectacular, with 5 petals
Calyx	Sepals are imbricate or valvate, 5, rarely 4, small, frequently glandular at the base, gamo- or polysepalous, and deeply lobed
Corolla	Five gamopetalous petals that can form a long or short corolla tube are present, and the petals are typically twisted, occasionally imbricate, and very rarely valvate
Androecium	The stigma is connate to the short, free, dithecous filament, which introrses in the 4 to 5 stamens and dehisces longitudinally
Gynoecium	When the ovaries are free, each ovary is unilocular with marginal placentation; when the ovaries are united, axile placentation occurs. The stigma is thickened distally. Ovaries that are free above but connected by the superior style alone are bicarpellary and syncarpous
Fruit	The fruit, which is made up of two follicles, splits along one side to release the seeds
Floral Formula	Br brl ⊕ ⚥ K5 C(5) A5 G(2)

1.3 **SECONDARY METABOLITES**

The intermediaries and by-products of metabolism are called metabolites. Small molecules are frequently categorised as metabolites. Fuel, signalling, structure, stimulating and inhibiting effects on enzymes, catalytic activity like an enzyme cofactor, resistance, and interconnections with other species are just a few of their many activities. The bulk of organic compounds produced by plants don't seem to be connected in any way to growth and development. These substances, which are typically referred to as secondary metabolites, are frequently distributed differently across the plant world [7].

- Alkaloids: Alkaloids are initially defined as basic compounds with nitrogen that are produced by plants and are pharmacologically active. They can also inhibit enzymes, close ion channels, and interfere with neurotransmission, which can result in hallucinations, coordination loss, convulsions, vomiting, and even death [8].
- Phenolics: Phenols are perhaps the largest class of secondary metabolites discovered in plants. Phenolics can stop cell division, impair enzyme activity, inhibit growth, or just taste awful. Based on their structural properties, phenolics can be divided into numerous categories, including simple phenolics, tannins, flavonoids, xanthones, lignans, and others [8, 9].

- Terpenes: Terpenes are one of the most prevalent and chemically varied families of natural substances. The naturally occurring class of chemicals known as terpenes has hydrocarbon bases and may share structural similarities with isoprene. The classification of terpenes is established on the quantity of five-carbon units. In plants, terpenes play a variety of physiological and ecological roles, such as allelophathy, insecticidal, insect pollinator, and plant hormone [9, 10].
- Flavonoids: More than 4500 unique examples of the flavanoids, a large family of phenolic natural chemicals, have now been identified. The majority of plant tissues contain flavonoids, which can be found as monomers, dimers, and higher oligomers, typically in vacuoles. A large class of compounds known as flavonoids has many different uses. Furthermore, certain flavonoids can protect plants from UV-B rays. The numerous kinds of plant metabolites known as flavonoids include chalcones, flavanones, isoflavonoids, flavonols, catechins, anthocyanins along with others [8, 10].
- Saponins: These are compounds that contain either a steroid or triterpenoid along with a polycyclic aglycone component attached to a carbohydrate unit. Pentoses, hexoses, or uronic acids, among other things, can be used to make these sugar units. Saponins have been found in more than 500 plants from at least 90 distinct families; they can be found in many various plants parts, including bulbs, leaves, stems, roots, flowers, and fruits, but they are typically concentrated in the roots of many species [9].
- Carbohydrates: Every living creature on Earth has carbs. The most common carbon-based matter on Earth is cellulose, which is a copolymer of glucose that acts as the chief constitutional element of plants. Carbon, hydrogen, and oxygen are the three components that make up carbohydrates, and these last two are often present in the same ratios as in water. Monosaccharides, disaccharides, oligosaccharides, and polysaccharides are the four chemical classes into which they are divided [10].
- Coumarins and Stilbenes: The benzopyranone family of plant metabolites, which has more than 1500 members in more than 800 species, includes coumarins as one of its many members. Plants have these chemicals in their seed coats, roots, leaves, fruits, flowers, and stems, however in general, the concentrations are higher in the fruits and flowers. Given their antibacterial, UV-screening, and germination inhibitory capabilities, their functions in plants appear to be mostly defense-related [9, 10]. Stilbenes are found in the bryophytes, pteridophytes, gymnosperms, and angiosperms,

and more than three hundred different stilbenoids have previously been described. The stilbenes are significant in pharmacology and human health as well as playing vital roles in plants, particularly in the protection of heartwood [8, 10].

1.4 **IN-SILICO STUDY**

A neurodegenerative ailment with a high mortality rate is Alzheimer's disease. This illness has a global impact of about 50 million people. In its most advanced phases, this illness results in patients losing their cognitive skills, memories, and brain cells to the point where they are entirely reliant on other people for survival [11]. There are several negative effects associated with current medications, and researchers are looking towards pharmaceuticals from natural sources. The medicinal plants *Hibiscus rosa-sinesis* and *Nerium oleander* Linn are the sources of several of the natural substances that are considered at in this study, which examines their anti-butyrylcholinesterase action.

A set of data containing information on the ligand or medication to be docked as well as the protein targets to be employed is required when molecular docking is performed. In silico molecular docking studies of chemical medicinal composites or bioactive peptides that function by engaging with specific receptors provide evidence regarding binding shape, pattern, and affinity. [12].

The Protein Data Bank (PDB) of the Research Collaboratory for Structural Bioinformatics (RCSB): Supporting international scientific research and education, the RCSB PDB makes it simpler to access annotated data about the three-dimensional (3D) structures of proteins, nucleic acids, and other macromolecules, as well as their associated small molecules, such as drugs, cofactors, and inhibitors, in the PDB archive. These databases are used to research the composition, relationships, and effects of biological molecules on molecular biology, biotechnology, and other domains. The development of standards for the atomic structural data that is saved, characterized, interpreted, and confirmed using a range of experimental approaches is made easier by the RCSB PDB. This website has tools for discovering, visualising, and studying PDB data [13].

PubChem: PubChem is a database that the general public can access to learn more about chemical compounds and their biological effects. Substance, Compound, and BioAssay are three interconnected databases that make up PubChem. Individual PubChem data contributors have provided chemical information to the Substance database, and Compound database has taken specific compound structures from the Substance database.

Information on biological activity of substances that have undergone assay testing can be found in the BioAssay database [14].

SwissADME: To estimate each compound's specific ADME behaviours, Swiss ADME software was employed. The list, which comprises one input molecule per line with numerous inputs, was made using SMILES. The findings are shown for each input molecule in tables, graphs, and an excel spreadsheet. For being successful as a drug, a potential particle needs to stay at the target site of the body in a bioactive form for a sufficient amount of time for the anticipated biologic processes to occur [15]. When there are many possible compounds but limited physical sample access, it is important to assess ADME at progressively previous phases of the discovery method in order to create new medications. Free access to a collection of quick but reliable predictive models for physicochemical properties, pharmacokinetics, drug-likeness, and medicinal chemistry friendliness is provided by the SwissADME [16].

BIOVIA Discovery Studio: In the rational development of small molecule pharmaceuticals, ligand-based design techniques are already well-established. These include virtual screening and the production of pharmacophore models. To develop and optimise compound selection, users of BIOVIA Discovery Studio can see, profile, and analyse various sources of chemical libraries. In BIOVIA Discovery Studio, there are also additional comprehensive and scalable tools for lead optimisation, virtual screening, and hit and lead identification [17].

Protein- Ligand Docking: The analysis of molecular interactions is based on molecular modelling. This molecular modelling objective is to forecast the shape of a ligand, the tiny molecule that binds to a protein receptor or enzyme. It is essential for the creation of pharmaceuticals and the discovery of new medicines since it can see the positions and orientations of molecules, proteins, and enzymes. Docking techniques are used in therapeutic research for a variety of purposes, most notably the virtual screening of enormous databases of compounds for the right subject [18]. Swiss Dock, a programme used for the same purpose, can calculate the shape, location, and energy of small molecules or peptides that interact with proteins.

Swiss Dock: It is a software programme for protein ligand docking that is free to use and is based on EADock DSS. The chemical interactions that take place between a small molecule and the large targeted protein can be seen using this free web tool [19]. It can

calculate all of the morphology and properties of the peptides and small molecules that interact with proteins, including their geometry, sites, and energies.

UCSF Chimera: This non-profit website resource is identified by the initials UCSF. The name Chimera is also used frequently. It is a flexible package that enables the addition of new abilities in order for it to work effectively. It is a tool for interactive molecular structure analysis and visualisation. It can also be used to look up data on docking results and their trajectories. Chimera can display the SwissDock predictions. A user-friendly web service called SwissDock targets a large technical research team for the docking of cutting-edge proteins and tiny compounds [20].

1.5 **OBJECTIVES OF STUDY**

The main objectives outlined are to implement:

- **Phytochemical investigation of chosen medicinal plants**
  a) Phytochemicals screening of medicinal plant *Hibiscus rosa-sinesis* by performing qualitative tests.
  b) Phytochemicals screening of medicinal plant *Nerium oleander* Linn. by performing qualitative tests.
- **Computational validation studies**

  In silico studies on molecules predicted from the chemical compounds of *Hibiscus rosa-sinesis* and *Nerium oleander* Linn. plants as ligands with potential targets for performing the protein-ligand molecular modelling and docking.

The following steps of work are proposed for apprehending the objectives:

- Collection of medicinal plants
- Plant Extract Preparation
- Phytochemical profiling of the chosen medicinal plant *Hibiscus rosa-sinesis* and *Nerium oleander* Linn.
- In silico studies on molecules predicted from the chemical compounds of *Hibiscus rosa-sinesis* and *Nerium oleander* Linn.
- In silico studies with targets and ligands and then perform the protein-ligand molecular modelling and docking.
- Preparation of the output files with the help of the application in order to retrieve interactions and analyse it.

1.6 **ORGANIZATION OF THE DISSERTATION**

In order to further describe the special aspects of phytochemical screening that are the subject of this thesis, the background information presented in the first section of this chapter includes an overview of fundamental introduction, medicinal plants, as well as secondary metabolites that are found in the selected medicinal plants. An outline of in-silico molecular docking for executing the target-ligand interactions is explained in the second section of this chapter.

Chapters 2 explains the basic introduction of the literature of selected medicinal plants and reviews about the medicinal plants with their chemical constituents, phytochemical profile and medicinal potential. The second part of the chapter reviews about the neurodegenerative disease for the In-silico study.

Chapter 3 explains the procedures and techniques utilised for the collection, extraction, and phyto-chemical profiling of different chemicals found in the selected medicinal plants. The in-silico molecular docking procedure for targets and ligands, which is utilised for obtaining and analysing interactions, is described in the second section of the chapter.

Chapter 4 discusses and analyses the results of the methods that are performed in the chapter 3 (i.e. phytochemical screening and in-silico molecular docking)

Chapter 5 lastly discusses and conclude the analysis and research work that is done in the dissertation by deliberating its future scope.

CHAPTER 2 REVIEW OF LITERATURE

A brief analysis of the literature is provided in this chapter in regard to the current study, which incorporates two medicinal herbs. For the plants, a thorough literature search was conducted.

Plant I: *Hibiscus rosa-sinensis* was reviewed.

Plant II: *Nerium oleander* Linn. was reviewed.

2.1 REVIEW LITERATURE ON *Hibiscus rosa-sinensis*

Hibiscus rosasinensis is sometimes referred to as the "China rose" as this plant is mostly found in South-East China and in Pacific and Indian Oceans limited islands. This plant is a vascular seed producer that belongs to the class Magnoliopsida and the subkingdom Magnoliophyta. It is a member of Malvaceae family and is one of the three hundred species of the *Hibiscus* genus [21]. The juice made from the leaves and blossoms has also been used for many years in herbal cosmetics and as a natural remedy for a wide range of illnesses and painful symptoms. In Hawaii it is commonly used for ceremonial hairstyles. Hibiscus is a popular major ingredient in herbal drinks and also has medicinal properties. Red-flowered hibiscus plants are advised in medicine. More than 50% of today's therapeutic drugs, according to recent scientific research, are derived from natural products. Many of them have made significant contributions to the pharmaceutical industry and the creation of more effective treatments for various diseases. Due to its use in herbal products and therapeutic purposes, this plant is extremely important commercially [21, 22]. Several studies have suggested that various hibiscus plants provide a range of medicinal benefits. The usage and potential health benefits of the *Hibiscus rosa sinensis* plant were the main focus of this review.

2.1.1 CHEMICAL CONSTITUENTS

A variety of chemicals can be found in every section of *H. rosasinensis*. According to reports, leaves, flowers, stems, and roots include glyco-sides, saponins, flavonoids, terpenoids, thiamine, niacin, and riboflavin. According to research on *H. rosa sinensis*, the presence of these chemicals enhanced the plant's pharmacological effects. [22]. The leaves of *Hibiscus rosa-sinensis* include carotene, fatty acids, alcohols, and hydrocarbons

like undecanoic, tridecanoic, tricosanoic, margaric, lignoceric and lauric acids. Additionally, gentisic acid and catalase have reportedly been found in petals and leaves. Flowers, leaves, and stems all comprise trace amounts of cyanins and cyanidin-chorides. But only the stems and leaves contain teraxeryl-acetate, malvalic-acids, and beta-sitosterol. There have been reports of quercetins, cyclopeptide-alkaloids, and hentriacontanes in the whole plants [23, 24].

2.1.2 **PHYTOCHEMICAL PROFILE**

To investigate the content of steroids, carbohydrates, glycosides: flavonoid, lipids, and alkaloids, preliminary photochemical screening was conducted.

2.1.3 **MEDICAL POTENTIAL**

- Antifertility Activity: The artificial birth control approach, hormonal contraceptives, is now used by more than 100 million women globally. This artificial way of preventing pregnancy has been shown to have a no of negative side effects in the majority of users, notwithstanding this fact. Winter-collected flowers had the strongest post-coital antifertility effects. Female humans consume dried flower ethanol extract orally. However, research has revealed that, when used as directed, *Hibiscus rosa-sinensis* contains anti-fertility qualities that make it effective as a natural contraceptive with no negative effects [25].
- Anti-inflammatory Activity: Rats were given an intraperitoneal dosage of dried leaf ethanol extract that prevented carrageenin-induced pedal edoema. The plant extract demonstrated strong anti-inflammatory effects against all the agents employed at the dose level when taken orally [26].
- Antioxidant activity: Since methanolic extract of flowers had a higher concentration of phenolics and flavonoids and had a stronger scavenging activity than ethanolic extract, it is highly likely that these compounds are what is causing the anti-oxidant activity. Ethanolic extract of flowers strongly scavenged hydrogen peroxide. Antioxidants are compounds that prevent free radical damage and control its effects. They are normally made available to the body through the natural foods that is regularly consumed. Antioxidants found in abundance in the hibiscus plant work to counteract free radicals and safeguard the immune system. Additionally, it stops free radicals from harming the body's other cells. High antioxidant concentrations

frequently have a positive role in the process and successfully stop the development of cancer cells and tumour growths [27].

- Anticancer activity: Oil from *H. rosa sinensis* was used to treat oral cancer cell lines. Oral cancer cells were unable to grow and multiply when treated with hibiscus extract. According to phytochemical study, the presence of terpenoids and flavonoids in the leaves was the primary factor controlling this potent anticancer impact. This anti-cancer activity is thought to be caused by the presence of phytochemicals like flavonoids, tannins, and saponins [28].
- Hair Growth Progression: Using in vivo and in vitro techniques, the petroleum ether extract of the plant's leaves and flowers was evaluated for its capacity to promote hair development. The leaf extract is more effective at promoting hair growth when compared to blossom extract [29].
- Antidiabetic activity: *Hibiscus rosa sinensis's* alcoholic leaf extracts have been shown to be effective oral hypoglycemics. The investigated extracts also markedly reduced triglyceride, glycosylated haemoglobin, blood urea, and cholesterol levels after weeks of oral dosage. Investigations were also conducted on the *H. rosa sinensis* ethanolic leaf extracts' potential ability to treat rats with alloxan-induced diabetes [24]. Using a methanolic extract of leaves, diabetic rats induced with streptozotocin had their blood sugar levels reduced. H&E histological analysis revealed that it reduced uric acid, creatinine, AST, and ALT concentrations, indicating protective effects on the kidneys and liver. In clinical trials, the powder from the flower was administered to type II diabetes mellitus patients between the ages of 30 and 60 to assess its potential as an anti-diabetic. Fasting blood glucose levels decreased following days of regular oral dosing. Additionally, significant reductions in triglyceride and total cholesterol levels were produced by the flower's powder. The aqueous extract's phytochemical examination revealed that saponins and steroids may have played a role in this anti-fertility effect. Similar to this, progesterone and oestrogen levels were decreased in pregnant female albino Wister rats by *H. rosa sinensis* flower extracts [24, 30].

2.2 **REVIEW LITERATURE ON *Nerium oleander* Linn**.

Nerium oleander L. is a tiny, geographically and ecologically diverse, evergreen tree that grows 2 to 5 meters tall. This plant is native to the Indian and Pakistani subcontinents and the Mediterranean region. Due to its abundant, long-lasting flowers and moderate hardiness, *Nerium oleander* is a common ornamental plant in tropical, subtropical, and

temperate climates. The Family Apocynaceae includes the drought-tolerant *Nerium oleander*. Screens, hedges along highways, and planting along coastlines are all uses for it. By leaving only a few stems, it can grow pretty little trees. It can be grown as an indoor or patio plant in northern latitudes. The plant also shown antibacterial, antimicrobial, anti-spasmodic, antinociceptive, as well as anticancer activities in addition to these [31]. Leprosy and alopecia were two persistent skin disorders that the leaves were used topically to treat. In order to treat epilepsy, leaf powder was snuffed. Haemorrhoids and sexual diseases were treated with root that had been pulverised and combined with water [31, 32].

2.2.1 CHEMICAL CONSTITUENTS

According to the initial phytochemical screening, the plant contained alkaloids, carbohydrates, cardiac glycosides, flavonoids, phenolics, tannins, saponins, triterpenoids, and triterpenes among many others. Cardenolides, pregnanes, and pregnane glycosides were all present in the plant. The roots contained the most oleandrin, followed by the leaves, stalks, and flowers. Triterpenoid compounds such as alpha- and beta-neriursates, oleanderolic acid, kanerodione, and ciskarenin, among others, were isolated from *Nerium oleander* leaves [32, 33]. Triterpenes of the taraxasterane and ursane types were extracted from an ethyl acetate preparation of Nerium oleander leaves. The oil produced by the flowers contains nériine, amorphane, limonene, and other key compounds. The primary component of the fraction existed a pectic-polysaccharides, mostly consisting of galacturonic acids, arabinose, galactose, and rhamnose. The ethanolic extract of the Nerium oleander flower contained a sub-extract of ethyl acetate that was used to separate chlorogenic acid, kaempferol, and kaempferol 3-O-glucopyranoside [33, 34].

2.2.2 PHYTOCHEMICAL PROFILE

Terpenoids, alkaloids, glycosides, saponins, tannin, and carbohydrates were discovered during the phytochemical screening. Phlobatanins, flavanoids, and phenolic substances all produced negative findings in the earlier studies [34].

2.2.3 MEDICAL POTENTIAL

- Antibacterial activity: *Nerium oleander* leaves had minimal antibacterial activity against gram positive *E. coli* but major activity against gram positive *Bacillus subtilis* in specific benzene and ethanol extracts. Comparative testing of the two extracts' antibacterial efficacy with the common antibiotic Ofloxacin revealed that ethanolic

extract had a relatively higher zone of inhibition than benzene extract, suggesting that it might be more effective against *Bacillus subtilis* [34]. The results showed that methanolic extract was superior to chloroform or hexane extract in terms of efficacy. Hepatoprotective and antioxidant activities of the *Nerium oleander* flower's methanolic extract have been demonstrated. [34, 35].

- Antidiabetic activity: Investigations were done into this plant's antidiabetic abilities. Both the defined mode of action and the particular physiologically active components in control for the anti-diabetic activity have not been described. They discovered that a single dose of *Nerium oleander* ethanolic extract dramatically lowered blood glucose levels as compared to the seventh day of their trial. After an hour, the chloroform extract significantly reduced blood sugar levels, while the ethanolic extract did so after three hours. On the glucose tolerance level in normal rats, the effects of several solvent extracts were studied. From the fasting value, the blood glucose level peaked quickly and then quickly decreased. The chloroform extract of *Nerium oleander* showed the highest glucose tolerance, while the aqueous extract showed the lowest glucose tolerance. When *Nerium oleander* activity was measured in chloroform extract, it was shown that diabetic rats had lower body weights and higher blood glucose levels than normal rats. In addition, oral administration of ethanolic and chloroform extracts of *Nerium oleander* dramatically boosted body weight and lowered blood glucose levels in diabetic rats [36].
- Antiproliferative activity: *N. oleander* leaf extracts have antiproliferative and phytochemical properties that were effective against MCF-7 cell lines.Sulforhodamine B test was used to measure the antiproliferative effects [32].
- Anticancer activity: The antiproliferative and phytochemical characteristics of *N. oleander* leaf extracts were effective against MCF-7 cell lines.The antiproliferative properties were evaluated using the Sulforhodamine B test [37].
- Hepatoprotective and antioxidant activity: The hepatoprotective, antioxidant, and other qualities of *Nerium oleander* methanolic floral extract were investigated against CCl4-induced hepatotoxicity in rats. The extract effectively provided the best hepatoprotection while helping to normalise the increased serum levels of the liver enzymes like Total bilirubin, aspartate-aminotransferase, alanine-aminotransferase, and alkaline-phosphatase. The biochemical proof of hepatoprotection was supported by the histological findings. Increased levels of SOD and a decline in MDA further

supported the hepatoprotective findings. *Nerium oleander* flower methanolic extract demonstrated liver-protective and antioxidant properties [38, 39].

2.3 ALZHEIMER'S DISEASE

Alzheimer's disease is one of the most common factor contributing to dementia. After a period of modest memory loss, the condition progressively gets worse, possibly leading to speech loss and a loss of feeling for one's surroundings. This disease affects the brain regions in charge of thought, memory, and language. It may significantly reduce the capacity to carry out daily tasks. The classic signs of Alzheimer's disease are still thought to include brain plaques and tangles. Another hallmark is the breakdown of connections between neurons in the brain [40]. Neurons provide impulses to the muscles, organs, and other parts of the brain. There are many more complex brain changes that could be a factor in Alzheimer's. Although the interval between diagnosis and death can vary. It might only take three or four years if the patient is under the age of 80 when they are diagnosed, but if they are younger, it might take ten years or more [41]. AD currently has no known cure, however research in this field has made significant strides in recent years. Several drugs have been given the go-ahead by the US Food and Drug Administration to treat Alzheimer's disease.

2.3.1 HALLMARKS

It has been found that acetylcholinesterase, β-amyloid, reactive oxygen species, tau protein, and metal ions are among the symptoms linked to the degenerative progression of the illness. There was agreement that additional pathogenic pathways, such as neuroinflammation, as well as the amyloid and tau pathologies should be targeted in AD research. The synthesis and accumulation of beta-amyloid protein in neuritic plaques and protein tau in neurofibrillary tangles are both caused by molecular pathways that these medications have been proven to be effective against [41, 42].

The cholinergic nerve system contains the enzymes AChE and BuChE. Acetylcholinesterase is a crucial enzyme that breaks down the neurotransmitter acetylcholine. Acetylcholinesterase is needed to hydrolyze acetylcholine into acetic acid and choline in a healthy brain, however in Alzheimer's disease, this process becomes troublesome. Acetylcholine levels are low, and they will continue to decline if acetylcholinesterase is not blocked. AChE and beta-amyloid may directly interact to encourage plaque development. AChE degree in the region of amyloid plaques and NFT

significantly increase over the course of the disease [42, 43]. The PAS at the canyon's mouth and the CAS at the canyon's bottom are AChE's two different binding sites within the enzyme gorge. Acetylcholinesterase inhibitors attach to one or more of these sites on the enzyme to reduce its action and lower the degree of acetylcholine breakdown, which enhances cholinergic neuro-transmission.. The medical AChE inhibitors that are now on the market have been demonstrated to enhance cognitive performance, but none of them have been exposed to suspend or stop the development of AD, underscoring the disease's complex pathophysiology. Blood and brain synapses are the main locations where AChE is found. The liver is where you'll typically find BuChE. The substrates are where the two diverge most. Acetylcholine (ACh) is hydrolyzed more quickly by AChE than is butyrylcholine (BuCh) by BuChE. A synthetic substance called BuCh is utilised to discriminate between BuChE and AChE receptors [43, 44, 45]. The majority of medications used to treat AD work by targeting both AChE and BuChE, however some are more selective than others.

While reviewing the prior literature, it was discovered that numerous studies had been conducted to assess the binding energies of numerous compounds with (BChE) in order to identify an effective ligand and inhibitor. Thioflavin T, which is one of the well-known inhibitors of Butyrylcholinesterase (BChE), along with many other inhibitors. Thioflavin T' is taken as a control ligand in the study [45]. However, there are very few investigations on the chemicals that are of interest from these medicinal plants, *Hibiscus rosa-sinensis* and *Nerium oleander* Linn. In order to discover more effective approaches and to check the efficacy to treat Alzheimer's disease, the phytochemical compounds of these medicinal plants is used to target BChE in this study with the aid of in silico experiments.

CHAPTER 3 MATERIALS AND METHODS

The study refers to phytochemical profiling of particular two medicinal plants (*Hibiscus rosa-sinensis*, *Nerium oleander* Linn.). The research work's approach will be given in two phases, as indicated below:

Phase I: Phytochemical profiling of *Hibiscus rosa-sinensis* and *Nerium oleander* Linn. leaves

Phase II: In silico studies

3.1 LAYOUT OF STUDY

- **Phytochemical profiling of *Hibiscus rosa-sinensis* and *Nerium oleander* Linn. leaves**
- Systematic collection and pre-processing of *Hibiscus rosa-sinensis* and *Nerium oleander* Linn. leaves
- Extraction of plant material
- Phytochemical determination by qualitative phytochemical screening
- **In silico studies was done for**
- Selected chemical compounds reported earlier from *Hibiscus rosa-sinensis* and *Nerium oleander* Linn.
- For the in silico studies the target chosen is Butyrylcholinesterase (BChE)

3.2 GENERAL

3.2.1 CHEMICALS AND SOLVENTS USED

- Standard techniques were used for extraction of the plant material.
- The experiment's chemicals and solvents were Wagner's reagent, Iodine, Potassium iodide, KOH solution, glacial acetic acid, ferric chloride, Sulphuric acid (H2SO4), copper sulphate solution, ethanol, potassium hydroxide (KOH) pellets, sodium hydroxide (NaOH) solution, hydrochloric acid (HCl), Gelatin solution, sodium chloride (NaCl), chloroform, gallic acid, isopropyl alcohol, ammonia solution.

3.2.2 INSTRUMENTS USED

- Mortar and pestle
- Whatmann filter paper

- Funnels
- Spatula
- Beakers
- Droppers
- Test tubes
- Conical flasks
- Glass measuring cylinders
- Falcon Tubes
- Pipettes
- Test Tube Stands
- Heating mantle
- Stainless steel electrical water bath was used for heating purposes

3.3 STEPS INVOLVED IN PLANT COLLECTION

3.3.1 Plant Material collection

The fresh leaves of *Hibiscus rosa-sinesis* and *Nerium oleander* Linn. medicinal plants were collected from the Delhi Technological University campus in Rohini, Delhi, India. The samples were approved by Plant Biotechnology Laboratory, Department of Biotechnology.

3.3.2 Cleaning of plants

After the collection of plant materials the plants were properly washed and cleaned. After being separated from their stems, the leaves were cleaned and rinsed in distilled water. To achieve better results, cleaning needs to be done by hand.

3.3.3 Drying

The fundamental goal of drying is the removal of water content from the plants. The plants must be dried as soon as they are picked in order to avoid plant parts going bad. The specimens can be dried in two different methods. There are both natural and artificial drying processes. In the experiment, the drying process is carried out naturally.

Natural Process: One of the natural drying processes is sun-drying. The specimens were shade and air dried after being sun dried for three to five days to get rid of the moisture. However, the entire drying procedure can take a few weeks. The temperature and humidity determine the time.

3.3.4 **Powdering**

The samples were then crushed and made into a coarse powder by using mortar and pestle and then stored at a room temperature of 35◦ C in airtight bottles.

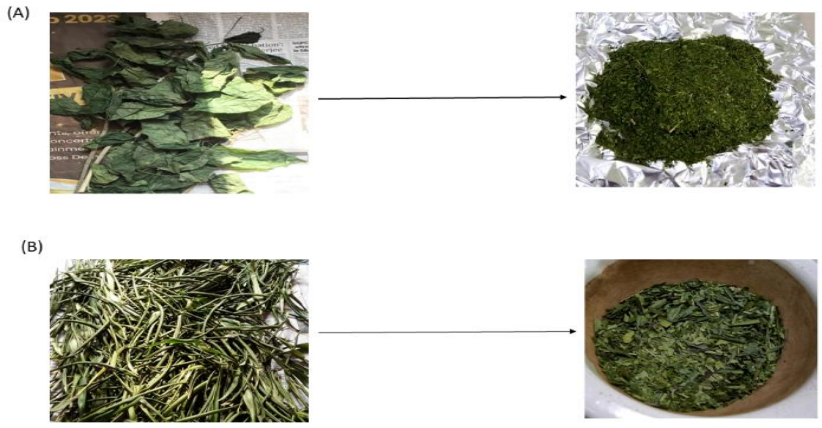


Fig 3.3.4.1: (A) *Hibiscus rosa-sinesis* and (B) *Nerium oleander* Linn. leaves before and after drying and powdering.

3.4 **EXTRACTION OF PLANT MATERIALS**

In two 500 ml flask, take 10 g of powder of each plant and mix it with 100 ml of milli-Q water before being heated on a heating mantle for approximately 10 minutes at 50 °C. The heated flask was then cooled to room temperature and the constituents were then transferred to a 50 ml falcon tube. The constituents of extract was then centrifuged for five minutes at 3000 rpm. The supernatant of the leaf pellets' was extracted out using a Whattman filter paper. The aqueous extract of the leaf is contained in the collected supernatant. The extract was then preserved in the refrigerator at 2-4◦ C for subsequent usage.

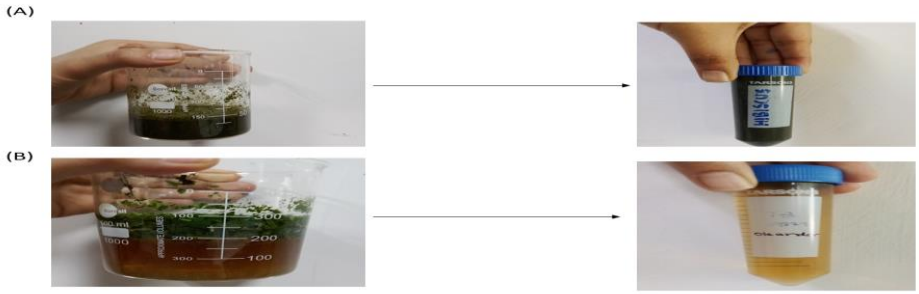


Fig 3.4.1: Aqueous extract of leaves (A) *Hibiscus rosa-sinesis* and (B) *Nerium oleander* Linn.

Phase I: Phytochemical profiling of leaves of *Hibiscus rosa-sinensis* and *Nerium oleander* Linn.

3.5 QUALITATIVE ANALYSIS OF THE PHYTOCHEMICALS

The following plants' extracts were put through a variety of chemical assays to identify various phytoconstituents using conventional procedures.

Table 3.5.1: Reagent Preparation for Screening of Phytochemical

Preparation of Wagner's Reagent	100mL of solution is created by combining distilled water with 1.27 grams of iodine (I) and 2 grams of potassium iodide (KI)

Table 3.5.2: Qualitative Phytochemical Screening Tests for *Hibiscus rosa-sinesis* and *Nerium oleander* Linn.

Test	PROCEDURE	REFERENCES
❖ TEST FOR ALKALOIDS Wagner's Test	2-5ml of plant extract filtrate is taken and then a small number of drops of Wagner's reagent is added along the walls of the test tube	[48, 52]
❖ TEST FOR ANTHRAQUINONES Borntrager's Test	2-5ml of filtrate is taken and then add 10mL 10% ammonia solution. Then vigorous shake it for 30 seconds	[47, 54, 55]
❖ TEST FOR CARBOHYDRATES Test for starch	2-5mL of aqueous extract is added to five ml of 5% KOH solution	[51]
❖ TEST FOR CARDIAC GLYCOSIDES Keller- Killani Test	In 2-5mL of filtrate add 1.5 mL of glacial acetic acid and 1 drop of 5% ferric chloride. Then concentrated H2SO4 is added along the side of the test tube	[52, 53]
❖ TEST FOR FLAVONOIDS Alkaline reagent test	In 2mL of extract add 2-4mL of 2% NaOH solution and little drops of dil. HCl	[51, 52, 53]
❖ TEST FOR LIGNINS Labat Test	In 2-5ml of extract solution add a little amount of gallic acid	[50, 56]
❖ TEST FOR PHENOLIC COMPOUNDS Ferric Chloride Test	In 2-5ml of extract's aqueous solution add a small number	[48, 49]

	of droplets of 5% ferric chloride solution	
❖ TEST FOR PROTEINS AND AMINO ACIDS Biuret Test	In 2-5ml of extract filtrate add 1-2 drops of 2% copper sulphate solution, then add 1 millilitre of 95% ethanol and some KOH pellets	[46, 48]
❖ TEST FOR SAPONINS Foam Test	In Ig of plant extract add 2mL of water and vigorously shake it	[49]
❖ TEST FOR TANNINS Gelatin Test	In 2-5ml of plant extract add 5mL of distilled water, then add about 1% gelatin solution and 10% NaCl	[49, 53]
❖ TEST FOR TERPINOIDES	First 2-5 ml of plant extract and 2 ml of chloroform is evaporated over water. Then add 3 ml of concentrated H2SO4 is added which is heated over water	[53]
❖ TEST FOR TRITERPINOIDES Salkowski's Test	In 2-5ml of filtrate add few drops of concentrated H2SO4. It should be fully shaken and left to stand	[52]
❖ TEST FOR QUINONES Sulphuric Acid Test	In 20mg plant extract dissolved in isopropyl alcohol add a few drops of concentrated H2SO4	[53]

Phase II: In silico studies

3.6 METHODS FOR IN-SILICO STUDIES

The following stages make up the in silico studies:

- **Selection of ligand**: Using internet resources like Google Scholar and NCBI, the different phytochemicals found in *Hibiscus rosa-sinensis* and *Nerium oleander* Linn. were looked for that might slow the progression of neurological disease. The PubChem database served as the source for all SMILES structures. Table 3.6.1 and Table 3.6.2 mentions the different phytochemicals that are found in the leaf part of the plant *Hibiscus rosa-sinensis* and *Nerium oleander* Linn. to predict ADMET parameters like water solubility, pharmacokinetics and drug likeness parameters using SwissADME.

Table 3.6.1: Different phytochemicals in *Hibiscus rosa-sinensis* with water solubility, pharmacokinetics and druglikeness parameters

Phytochemicals	Lipinski	Ghose	GI absorption	Class
Riboflavin	0	1	Low	Soluble
Cyanidin 3-sophoroside-5-glucoside	3	4	Low	Soluble
Malvalic acid	1	1	High	Moderately soluble
Quercetin 3-diglucoside	3	4	Low	Soluble
D-Glucuronic Acid	0	2	Low	Soluble
Quercetin	0	-	High	Soluble
Ascorbic acid	0	2	High	Soluble
Nicotinic acid	0	3	High	Soluble
Taraxerol acetate	1	3	Low	Poorly soluble
Hentriacontane	1	3	Low	Insoluble
Cyanidin chloride	0	-	High	Soluble
Sterculic acid	1	1	High	Poorly soluble
Kaempferol 3-xylosylglucoside	3	3	Low	Soluble
alpha-Carotene	2	4	Low	Poorly soluble
D-Galactose	0	2	Low	Soluble
beta-Sitosterol	1	3	Low	Poorly soluble
D-Galacturonic Acid	0	2	Low	Soluble
L-Rhamnose	0	2	High	Soluble

Table 3.6.2: Different phytochemicals in *Nerium oleander* Linn. with water solubility, pharmacokinetics and druglikeness parameters

Phytochemicals	Lipinski	Ghose	GI absorption	Class
Oleagenin	0	-	High	Moderately soluble
Adynerin	1	3	High	Moderately soluble
16-Dehydroadynerigenin-beta-d-diginoside	1	3	High	Soluble
Neriaside	1	3	High	Soluble
Oleandrin	1	3	High	Soluble
Digitoxigenin	0	-	High	Soluble
Odoroside K	3	3	Low	Soluble
L-(+)-Arabinose	0	3	Low	Soluble
Betulin	1	3	Low	Poorly soluble
Dambonitol	0	1	Low	Soluble
Oleandrigenin	0	-	High	Soluble

Kaneric acid	1	3	High	Moderately soluble
Neriantin	1	3	Low	Soluble
Oleanderol	1	3	High	Moderately soluble
Urs-12(13)-ene	1	3	Low	Poorly soluble
Desacetyloleandrin	1	3	High	Soluble
Urechitoxin	2	3	Low	Soluble
Isoneriucoumaric acid	2	4	Low	Poorly soluble
Bicuculline	0	-	High	Moderately soluble
Oleanolic acid	1	3	Low	Poorly soluble
Ursolic acid	1	3	Low	Moderately soluble
Betulinic acid	1	3	Low	Moderately soluble
Oxotremorine	0	-	High	Soluble
D-Galactose	0	2	Low	Soluble
Isoquercitrin	2	1	Low	Soluble
Methyl 8,16-dihydroxyhexadecanoate	0	2	Low	Soluble
Adynerigenin beta-neritrioside	3	3	Low	Soluble
C15H18O7.C15H16O6	2	4	Low	Soluble
Adynerigenine	0	-	High	Soluble
Neritaloside	1	3	Low	Soluble
beta-Sitosterol	1	3	Low	Poorly soluble
D-Galacturonic Acid	0	2	Low	Soluble
Solanoside	1	3	High	Soluble
Rutin	3	4	Low	Soluble
Quercitrin	2	1	Low	Soluble
L-Rhamnose	0	2	High	Soluble

- After following the different physiochemical parameters using SwissADME selection and shortlisting of the following phytochemicals of *Hibiscus rosa-sinesis* and *Nerium oleander* Linn plants are given in Table 3.6.3 and Table 3.6.4 as the ligands for in-silico studies.

Table 3.6.3: Different phytochemicals shortlisted in *Hibiscus rosa-sinesis* for in-silico studies.

Phytochemicals	Lipinski	Ghose	GI absorption	Class
Riboflavin	0	1	Low	Soluble
Quercetin	0	-	High	Soluble

Table 3.6.4: Different phytochemicals shortlisted in *Nerium oleander* Linn for in-silico studies.

Phytochemicals	Lipinski	Ghose	GI absorption	Class
Oleagenin	0	-	High	Moderately soluble
Digitoxigenin	0	-	High	Soluble
Dambonitol	0	1	Low	Soluble
Oleandrigenin	0	-	High	Soluble
Bicuculline	0	-	High	Moderately soluble
Oxotremorine	0	-	High	Soluble
Adynerigenine	0	-	High	Soluble

- **Retrieval of target**: For in-silico studies the target chosen is Butyrylcholinesterase (BChE)-**6EP4**. The PDB crystal 6EP4 has A chain and sequence length of 529AA. The protein Butyrylcholinesterase (BChE)-**6EP4** (target) was produced from BIOVIA Discovery Studio for the docking of the target protein with the selected compounds. The target is retrieved through the RCSB-PDB which enables approach to interpreted data on the three-dimensional (3D) structures of macromolecules. The protein target file was then saved in the PDB format.

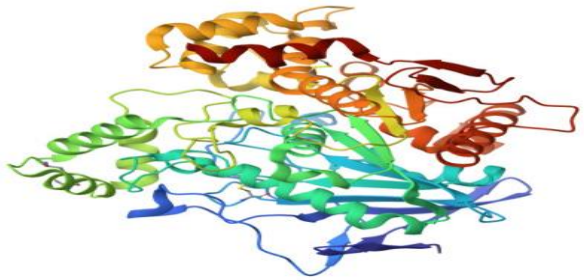


Fig 3.6.1: The 3-dimensional structure of Butyrylcholinesterase (BChE)-6EP4 target

- **Preparation of the target**: The target Butyrylcholinesterase (BChE) - 6EP4 proteins' 3D structures were acquired and used to build the protein using BIOVIA Discovery Studio. During protein preparation, bond ordering was allocated and hydrogen atoms

were also added. Hetero atom groups, water molecules were eliminated. The prepared protein structure of target protein that had been generated was then stored in PDB format and were used further for the docking studies.

- **Preparation of the ligand**: The ligand molecules were prepared using the BIOVIA Discovery Studio. The ligand SDF structures were converted into MOL2 format and was stored to be used further for the docking studies.

- **Molecular docking using Swiss Dock**: To examine the molecular interaction between several licenced medications and the target protein, blind docking was done using a SwissDock server in the accurate mode without flexibility of any of the target protein's side chains. Furthermore, a binding pocket wasn't specified to prevent docking from favouring the active site. By submitting PDB and Mol2 files, respectively, the protein and ligand were identified. Following the generation of entirely probable binding modes to each ligand by Swiss Dock, the utmost beneficial binding modes at a specified pocket was assembled. The ligand cluster information was saved in an output file named "prediction file". The file contained information on cluster rank, element, fitness, as well as projected binding free energy (G). The many conformations of a ligand inside a specific cluster are represented by the cluster rank, and the predicted binding pocket on the target protein is represented by a cluster. Only the cluster zero model with the lowest energy was deemed to have the best interaction. According to it, the compounds were chosen, and ligands with binding energies lower than the known inhibitor (control) were taken into account.

- **UCSF Chimera**: The output files with this application were used to retrieve the interactions between the protein (target) and ligands. The file in PDB format was saved.

- **Examining**: Now the saved PDB format file is opened in the BIOVIA Discovery Studio and the inhibitor showing ligand-protein interactions and 2D interactions are saved.

CHAPTER 4 RESULTS AND DISCUSSION

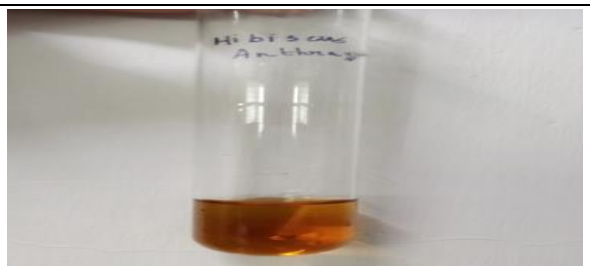
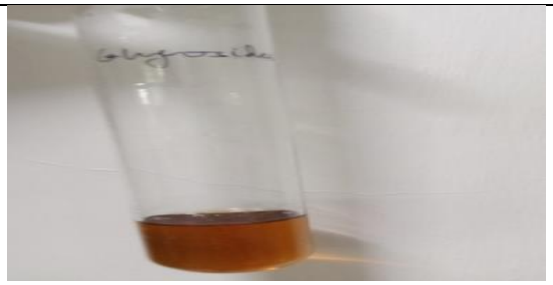
PHASE I: Phytochemical Profiling of Leaves of *Hibiscus rosa-sinesis* and *Nerium oleander Linn.*

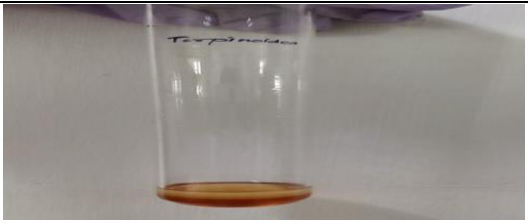
4.1 QUALITATIVE PHYTOCHEMICAL SCREENING TESTS RESULTS FOR *Hibiscus rosa-sinesis* and *Nerium oleander Linn.*

Table 4.1.1: Qualitative Phytochemical Screening Tests Results for *Hibiscus rosa-sinesis*

Test	Procedure	Expected Result	Observed Result
❖ TEST FOR ALKALOIDS Wagner's Test	2-5ml of plant extract filtrate is taken and then a small number of drops of Wagner's reagent is added along the walls of the test tube	Reddish-brown precipitate is seen	Positive
❖ TEST FOR ANTHRAQUINONES Borntrager's Test	2-5ml of filtrate is taken and then add 10mL 10% ammonia solution. Then vigorous shake it for 30 seconds	Pink, violet, or red coloured solution is seen	Negative
❖ TEST FOR CARBOHYDRATES Test for starch	2-5mL of aqueous extract is added to five ml of 5% KOH solution	Colouration of the cinary is seen	Positive
❖ TEST FOR CARDIAC GLYCOSIDES Keller- Killani Test	In 2-5 mL of filtrate add 1.5 mL of glacial acetic acid and 1 drop of 5% ferric chloride. Then concentrated H2SO4 is added along the side of the test tube	In acetic acid layer blue coloured solution in seen	Negative
❖ TEST FOR FLAVONOIDS Alkaline reagent test	In 2mL of extract add 2-4mL of 2% NaOH solution and little drops of dil. HCl	Strong yellow colouring turning into colourless is seen when diluted acid is added.	Positive

❖ TEST FOR LIGNINS Labat Test	In 2-5ml of extract solution add a little amount of gallic acid	Olive green colour is seen	Negative
❖ TEST FOR PHENOLIC COMPOUNDS Ferric Chloride Test	In 2-5ml of extract's aqueous solution add a small number of droplets of 5% ferric chloride solution	The colours seen is dark green or bluish black	Positive
❖ TEST FOR PROTEINS AND AMINO ACIDS Biuret Test	In 2-5ml of extract filtrate add 1-2 drops of 2% copper sulphate solution, then add 1 millilitre of 95% ethanol and some KOH pellets	Pink coloured solution in ethanolic layer is seen	Negative
❖ TEST FOR SAPONINS Foam Test	In 1g of plant extract add 2mL of water and vigorously shake it	Foam that lasted for about 10 minutes is seen	Positive
❖ TEST FOR TANNINS Gelatin Test	In 2-5ml of plant extract add 5mL of distilled water, then add about 1% gelatin solution and 10% NaCl	White colour precipitate is seen	Positive
❖ TEST FOR TERPINOIDES	First 2-5 ml of plant extract and 2 ml of chloroform is evaporated over water. Then add 3 ml of concentrated H2SO4 is added which is heated over water.	Grey-coloured solution is seen	Negative
❖ TEST FOR TRITERPINOIDES Salkowski's Test	In 2-5ml of filtrate add few drops of concentrated H2SO4. It should be fully shaken and left to stand	At the bottom layer golden yellow colour is seen	Positive
❖ TEST FOR QUINONES Sulphuric Acid Test	In 20mg plant extract dissolved in isopropyl alcohol add a few drops of concentrated H2SO4	Reddish colour is seen	Positive

TEST	RESULT
❖ TEST FOR ALKALOIDS Wagner's Test	 POSITIVE
❖ TEST FOR ANTHRAQUINONES Borntrager's Test	 NEGATIVE
❖ TEST FOR CARBOHYDRATES Test for starch	 POSITIVE
❖ TEST FOR CARDIAC GLYCOSIDES Keller- Killani Test	 NEGATIVE
❖ TEST FOR FLAVONOIDS Alkaline reagent test	 POSITIVE

❖ TEST FOR LIGNINS Labat Test	 NEGATIVE
❖ TEST FOR PHENOLIC COMPOUNDS Ferric Chloride Test	 POSITIVE
❖ TEST FOR PROTEINS AND AMINO ACIDS Biuret Test	 NEGATIVE
❖ TEST FOR SAPONINS Foam Test	 POSITIVE
❖ TEST FOR TANNINS Gelatin Test	 POSITIVE
❖ TEST FOR TERPINOIDES	 NEGATIVE

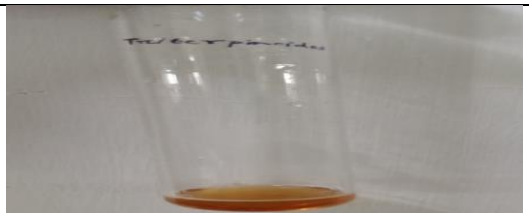
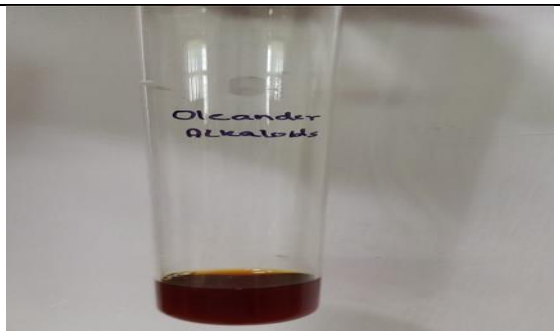
❖ TEST FOR TRITERPINOIDES Salkowski's Test	 POSITIVE
❖ TEST FOR QUINONES Sulphuric Acid Test	 POSITIVE

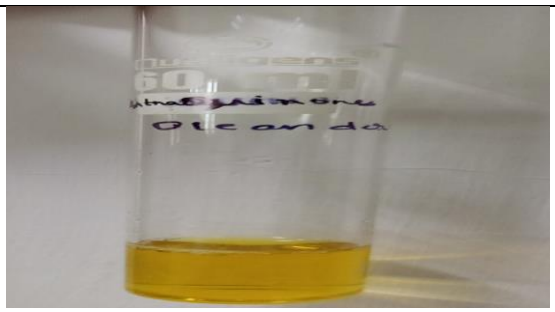
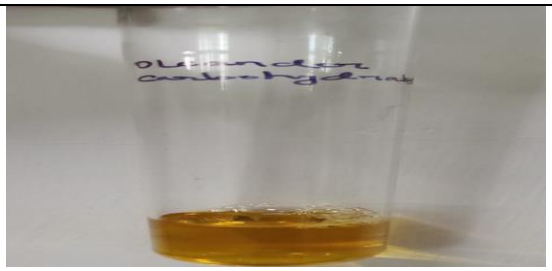
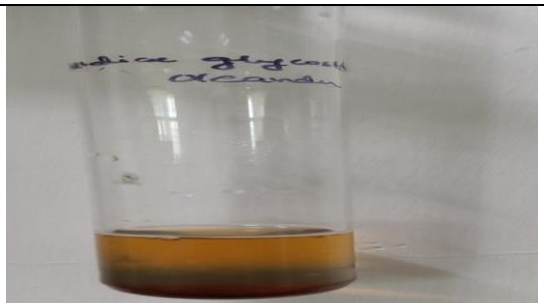
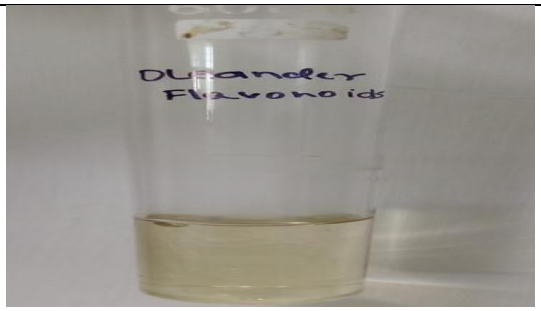
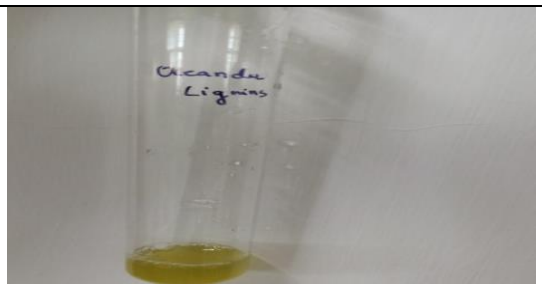
Fig 4.1.1: Qualitative Phytochemical Screening Tests Results for *Hibiscus rosa-sinesis*

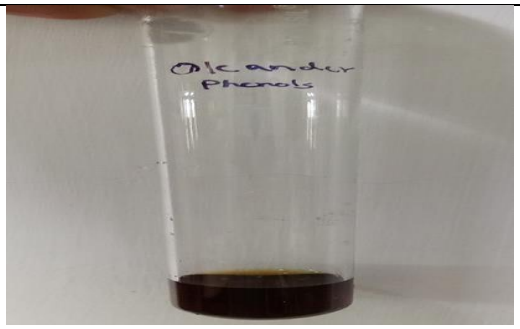
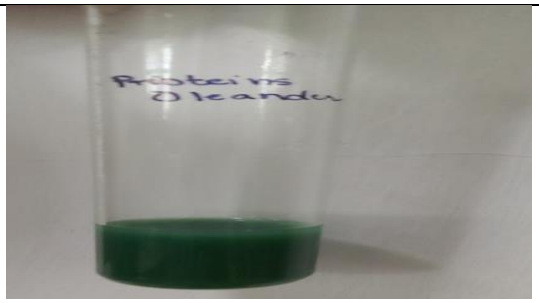
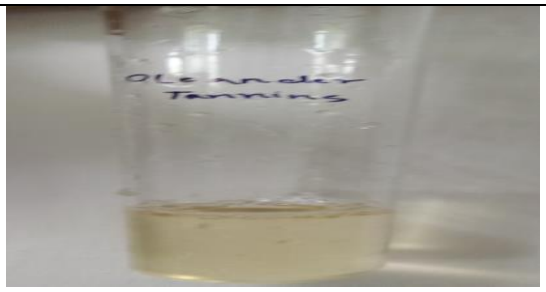
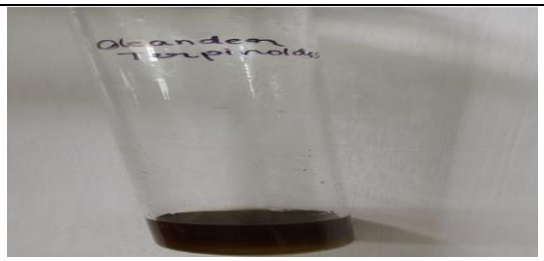
Table 4.1.2: Qualitative Phytochemical Screening Tests Results for *Nerium oleander* Linn.

Test	Procedure	Expected Result	Observed Result
❖ TEST FOR ALKALOIDS Wagner's Test	2-5ml of plant extract filtrate is taken and then a small number of drops of Wagner's reagent is added along the walls of the test tube	Reddish-brown precipitate is seen	Positive
❖ TEST FOR ANTHRAQUINONES Borntrager's Test	2-5ml of filtrate is taken and then add 10mL 10% ammonia solution. Then vigorous shake it for 30 seconds.	Pink, violet, or red coloured solution is seen	Negative
❖ TEST FOR CARBOHYDRATES Test for starch	2-5mL of aqueous extract is added to five ml of 5% KOH solution	Colouration of the cinary is seen	Positive
❖ TEST FOR CARDIAC GLYCOSIDES Keller- Killani Test	In 2-5 mL of filtrate add 1.5 mL of glacial acetic acid and 1 drop of 5% ferric chloride. Then concentrated H2SO4 is added along the side of the test tube.	In acetic acid layer blue coloured solution in seen	Positive
❖ TEST FOR FLAVONOIDS Alkaline reagent test	In 2mL of extract add 2-4mL of 2% NaOH solution and little drops of dil. HCl	Strong yellow colouring turning into colourless is seen when diluted acid is added.	Positive

❖ TEST FOR LIGNINS Labat Test	In 2-5ml of extract solution add a little amount of gallic acid	Olive green colour is seen	Positive
❖ TEST FOR PHENOLIC COMPOUNDS Ferric Chloride Test	In 2-5ml of extract's aqueous solution add a small number of droplets of 5% ferric chloride solution	The colours seen is dark green or bluish black	Positive
❖ TEST FOR PROTEINS AND AMINO ACIDS Biuret Test	In 2-5ml of extract filtrate add 1-2 drops of 2% copper sulphate solution, then add 1 millilitre of 95% ethanol and some KOH pellets	Pink coloured solution in ethanolic layer is seen	Negative
❖ TEST FOR SAPONINS Foam Test	In 1g of plant extract add 2mL of water and vigorously shake it	Foam that lasted for about 10 minutes is seen	Positive
❖ TEST FOR TANNINS Gelatin Test	In 2-5ml of plant extract add 5mL of distilled water, then add about 1% gelatin solution and 10% NaCl	White colour precipitate is seen	Positive
❖ TEST FOR TERPINOIDES	First 2-5 ml of plant extract and 2 ml of chloroform is evaporated over water. Then add 3 ml of concentrated H2SO4 is added which is heated over water.	Grey-coloured solution is seen	Negative
❖ TEST FOR TRITERPINOIDES Salkowski's Test	In 2-5ml of filtrate add few drops of concentrated H2SO4. It should be fully shaken and left to stand	At the bottom layer golden yellow colour is seen	Positive
❖ TEST FOR QUINONES Sulphuric Acid Test	In 20mg plant extract dissolved in isopropyl alcohol add a few drops of concentrated H2SO4	Reddish colour is seen	Positive

TEST	**RESULT**
❖ TEST FOR ALKALOIDS Wagner's Test	 POSITIVE

❖ TEST FOR ANTHRAQUINONES Borntrager's Test	 NEGATIVE
❖ TEST FOR CARBOHYDRATES Test for starch	 POSITIVE
❖ TEST FOR CARDIAC GLYCOSIDES Keller- Killani Test	 POSITIVE
❖ TEST FOR FLAVONOIDS Alkaline reagent test	 POSITIVE
❖ TEST FOR LIGNINS Labat Test	 POSITIVE

❖ TEST FOR PHENOLIC COMPOUNDS Ferric Chloride Test	 POSITIVE
❖ TEST FOR PROTEINS AND AMINO ACIDS Biuret Test	 NEGATIVE
❖ TEST FOR SAPONINS Foam Test	 POSITIVE
❖ TEST FOR TANNINS Gelatin Test	 POSITIVE
❖ TEST FOR TERPINOIDES	 NEGATIVE

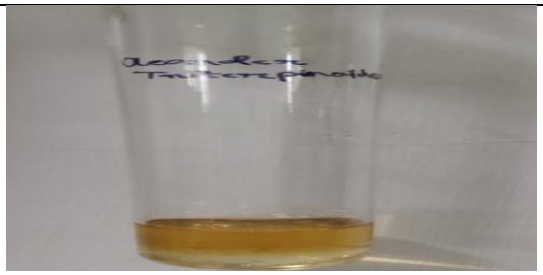
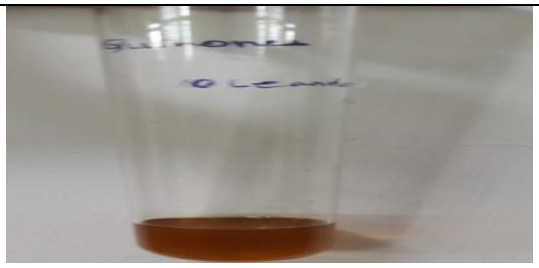
❖ TEST FOR TRITERPINOIDES Salkowski's Test	 POSITIVE
❖ TEST FOR QUINONES Sulphuric Acid Test	 POSITIVE

Fig 4.1.2: Qualitative Phytochemical Screening Tests Results for *Nerium oleander* Linn.

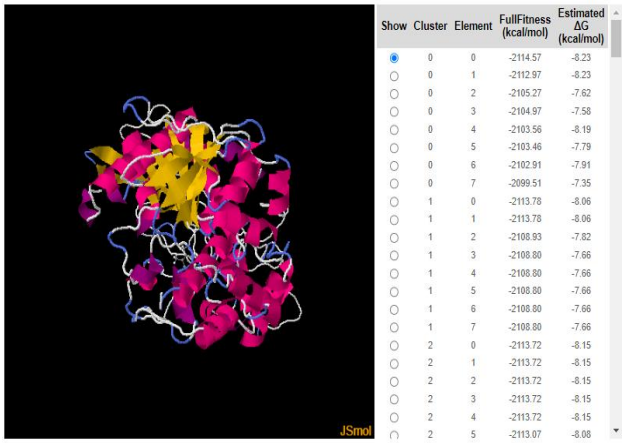
PHASE II: In silico Studies

4.2 **IN- SILICO STUDIES ON SELECTED COMPOUNDS OF *Hibiscus rosa-sinesis* and *Nerium oleander Linn.***

Table 4.2.1: Binding Energies of best inhibitors (ligands) against Butyrylcholinesterase (BChE)

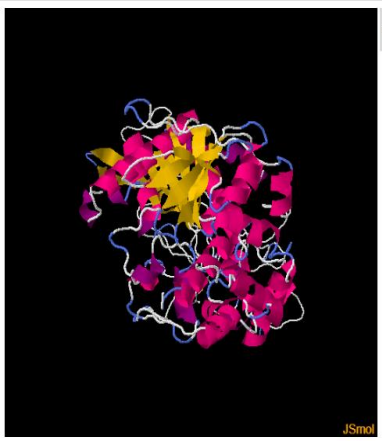
INHIBITORS	Binding Energy(kcal/mol)
Riboflavin	-8.23
Quercetin	-8.07
Oleagenin	-8.22
Digitoxigenin	-7.92
Dambonitol	-6.29
Oleandrigenin	-7.75
Bicuculline	-7.53
Oxotremorine	-7.28
Adynerigenine	-7.22

Docking results revealed that Riboflavin in *Hibiscus rosa-sinensis* and Oleagenin in *Nerium oleander* Linn. has the best energies of binding −8.23, -8.22 kcal/mol, respectively. Both of these compounds Riboflavin and Oleagenin satisfied the criteria of having a binding energy lower than the binding energy of Thioflavin T i.e. -7.90 kcal/mol. Thioflavin T is a well-known inhibitor of Butyrylcholinesterase (BChE).



Show	Cluster	Element	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
◉	0	0	-2114.57	-8.23
○	0	1	-2112.97	-8.23
○	0	2	-2105.27	-7.62
○	0	3	-2104.97	-7.58
○	0	4	-2103.56	-8.19
○	0	5	-2103.46	-7.79
○	0	6	-2102.91	-7.91
○	0	7	-2099.51	-7.35
○	1	0	-2113.78	-8.06
○	1	1	-2113.78	-8.06
○	1	2	-2108.93	-7.82
○	1	3	-2108.80	-7.66
○	1	4	-2108.80	-7.66
○	1	5	-2108.80	-7.66
○	1	6	-2108.80	-7.66
○	1	7	-2108.80	-7.66
○	2	0	-2113.72	-8.15
○	2	1	-2113.72	-8.15
○	2	2	-2113.72	-8.15
○	2	3	-2113.72	-8.15
○	2	4	-2113.72	-8.15
○	2	5	-2113.07	-8.08

Riboflavin inhibitor (ligand)



Show	Cluster	Element	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
◉	0	0	-2146.95	-7.90
○	0	1	-2146.95	-7.89
○	0	2	-2146.92	-7.89
○	0	3	-2144.17	-7.49
○	0	4	-2144.15	-7.46
○	0	5	-2144.12	-7.47
○	1	0	-2144.14	-7.49
○	1	1	-2144.09	-7.48
○	1	2	-2143.35	-7.47
○	1	3	-2143.35	-7.48
○	1	4	-2143.24	-7.48
○	1	5	-2141.96	-7.14
○	1	6	-2141.61	-7.14
○	1	7	-2141.37	-7.08
○	1	8	-2141.35	-7.10
○	1	9	-2141.35	-7.10
○	1	10	-2141.17	-7.11
○	1	11	-2141.17	-7.11
○	1	12	-2141.09	-7.11
○	1	13	-2141.09	-7.11
○	1	14	-2141.09	-7.11
○	1	15	-2141.09	-7.11

Thioflavin T (control)

Fig 4.2.1: Estimated binding energies of Riboflavin inhibitor (ligand) and Thioflavin T (control) are shown

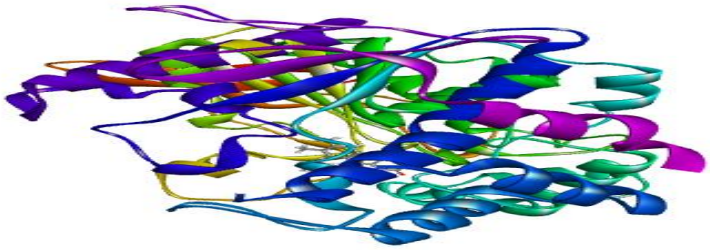


Fig 4.2.2: Riboflavin inhibitor showing ligand-protein interactions

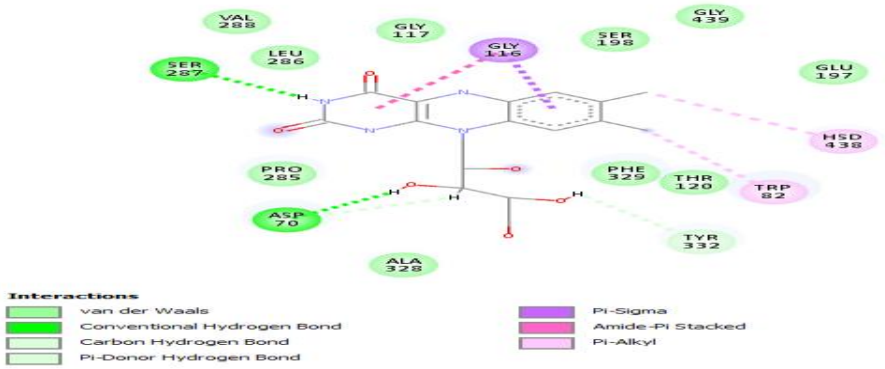
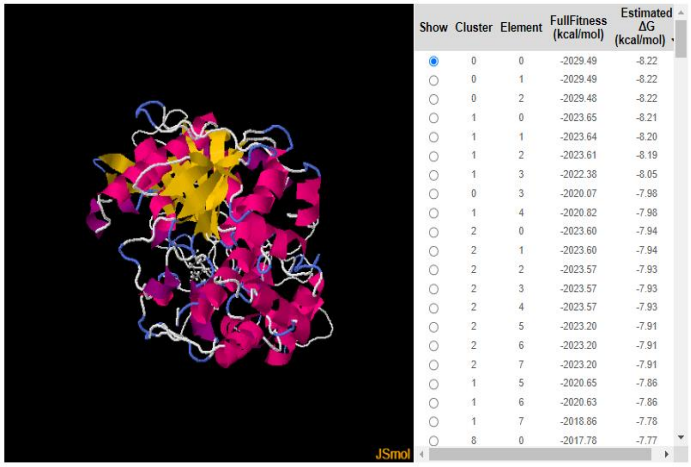
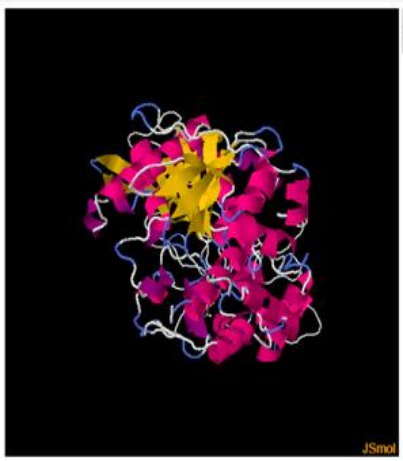


Fig 4.2.3: Riboflavin inhibitor showing 2D interactions



Show	Cluster	Element	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
◉	0	0	-2029.49	-8.22
○	0	1	-2029.49	-8.22
○	0	2	-2029.48	-8.22
○	1	0	-2023.65	-8.21
○	1	1	-2023.64	-8.20
○	1	2	-2023.61	-8.19
○	1	3	-2022.38	-8.05
○	0	3	-2020.07	-7.98
○	1	4	-2020.82	-7.98
○	2	0	-2023.60	-7.94
○	2	1	-2023.60	-7.94
○	2	2	-2023.57	-7.93
○	2	3	-2023.57	-7.93
○	2	4	-2023.57	-7.93
○	2	5	-2023.20	-7.91
○	2	6	-2023.20	-7.91
○	2	7	-2023.20	-7.91
○	1	5	-2020.65	-7.86
○	1	6	-2020.63	-7.86
○	1	7	-2018.86	-7.78
○	8	0	-2017.78	-7.77

Oleagenin inhibitor (ligand)



Show	Cluster	Element	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
◉	0	0	-2146.95	-7.90
○	0	1	-2146.95	-7.89
○	0	2	-2146.92	-7.89
○	0	3	-2144.17	-7.49
○	0	4	-2144.15	-7.46
○	0	5	-2144.12	-7.47
○	1	0	-2144.14	-7.49
○	1	1	-2144.09	-7.48
○	1	2	-2143.35	-7.47
○	1	3	-2143.35	-7.48
○	1	4	-2143.24	-7.48
○	1	5	-2141.96	-7.14
○	1	6	-2141.61	-7.14
○	1	7	-2141.37	-7.08
○	1	8	-2141.35	-7.10
○	1	9	-2141.35	-7.10
○	1	10	-2141.17	-7.11
○	1	11	-2141.17	-7.11
○	1	12	-2141.09	-7.11
○	1	13	-2141.09	-7.11
○	1	14	-2141.09	-7.11
○	1	15	-2141.09	-7.11

Thioflavin T (control)

Fig 4.2.4: Estimated binding energies of Oleagenin inhibitor (ligand) and Thioflavin T (control) are shown here

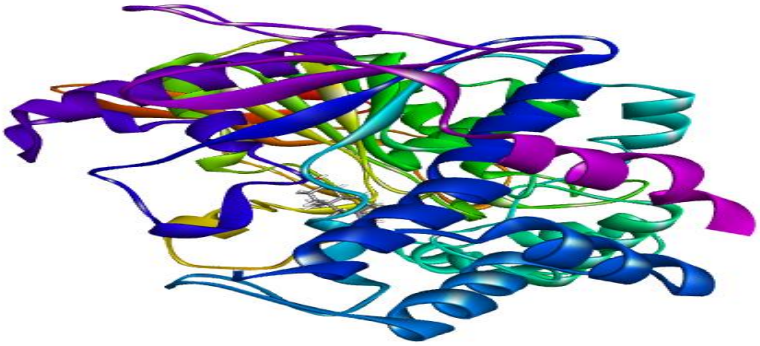


Fig 4.2.5: Oleagenin inhibitor showing ligand-protein interactions

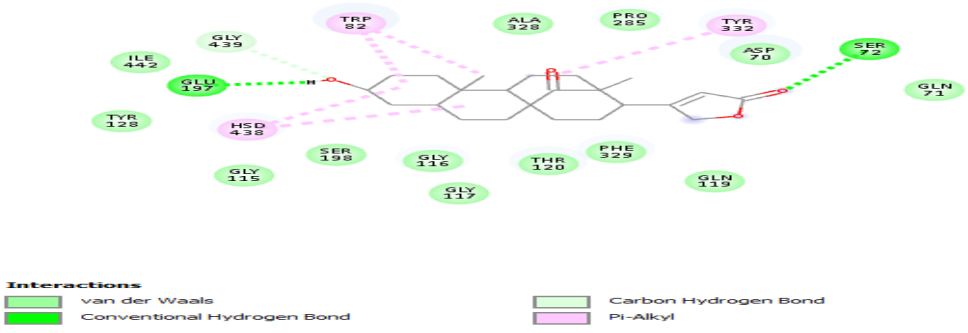


Fig 4.2.6: Oleagenin inhibitor showing 2D interactions

4.3 **DISCUSSION**

In the leaves of *Hibiscus rosa-sinesis* and *Nerium oleander Linn*, phytochemical components were found. Both plant samples' extracts passed a preliminary phytochemical screening, which identified a number of phytoconstituents including alkaloids, carbohydrates, glycosides, flavonoids, saponins, tannins, phenolic compounds, proteins and amino acids, and quinones. These phytoconstituents support the explanation for the wide range of biological activities. It was discovered that *Hibiscus rosa-sinesis* lacked cardiac glycosides and lignins. The BChE inhibitory activity of these plant species' extracts, which are employed as herbal remedies for various illnesses, was examined. Table 4.2.1 displays the binding energies of the top ligands (inhibitors) for Butyrylcholinesterase (BChE). Riboflavin and oleagenin were determined to have the lowest inhibitory activity. The effectiveness of the extracts against both cholinesterase (ChE) is essential since BChE is more common in the peripheral system. These discoveries may help people with Alzheimer's disease (AD) have better mental functioning by primarily activating central and peripheral cholinergic transmission. Out of the nine extracts selected for evaluation, riboflavin and oleagenin were determined to be strong contenders as sources of powerful ChE (BChE) inhibitors. Multitargeted medications will be preferred as the most efficient treatment for AD due to its complex pathophysiology. Parts of *Hibiscus rosa-sinesis* and *Nerium oleander* Linn. showed a variety of positive effects, including hypotensive, anti-inflammatory, antioxidant, anticancer, hepatoprotective, antifungal, antidiabetic, and antiHIV activity remedies. The cholinergic nerve system's primary enzymes are butyrylcholinesterase. According to several studies, cholinesterase inhibitors can affect a variety of therapeutic goals, including antioxidant activity, the control of APP processing, and the prevention of amyloid plaque formation. In silico molecular docking has shown to be a successful method in modern structure-based drug creation. Strong binding affinities were displayed by all of the ligands. The highest binding affinities with BChE were shown by riboflavin inhibitor i.e. -8.23 kcal/mol and oleagenin i.e. -8.22 kcal/mol. At the side chains of the ligands the hydroxyl groups play a crucial role in ligand-protein interactions by developing an H (hydrogen) bond with the residues of the protein. In contrast to AChE, which has several aromatic residues replaced by residues with aliphatic side chains, such as Leu and Val, BChE can handle larger molecules. The biological activity and binding affinity of natural substances are both reflected in their chemical structure. The target

Butyrylcholinesterase (BChE)-6EP4 with the inhibitors Riboflavin in *Hibiscus rosa-sinensis* ensured the binding affinity of -8.23 kcal/mol and Oleagenin in *Nerium oleander* Linn. ensured the binding affinity of -8.22 kcal/mol, respectively, according to the docking studies. These inhibitors serve both therapeutic and medical purposes by blocking the BChE pathway. These substances, riboflavin and oleagenin, met the requirement of having a binding affinity lower than that of Thioflavin T having a binding energy of -7.90 kcal/mol. The more is the negative energy the better is the ligand. Thioflavin T which is one of the well-known inhibitors of Butyrylcholinesterase (BChE), along with many other inhibitors like decamethonium, propidium, huprine, and ethopropazine. This inhibition and reduced binding energy lead to improved symptoms in Alzheimer's disease (AD) patients.

CHAPTER 5 CONCLUSION AND FUTURE SCOPE

Both *Hibiscus rosa sinensis* and *Nerium oleander* leaves included phenols, tannins, flavonoids, alkaloids, and saponins, according to the phytochemical screening done on them. The anti-inflammatory, bacterial infection, and even contraceptive effects of the extracts of medicinal leaves of *Hibiscus rosa sinensis* and *Nerium oleander* have been proven. The biological actions of these extracts are most likely caused by the phytochemicals that are present in them. These medicinal plant's lower toxicity makes them suitable for application as a new medicinal agent. This research study has provided a thorough overview of recent investigations into the phytochemistry and therapeutic applications of medicinal plants *Hibiscus rosa sinensis* and *Nerium oleander*. The occurrence of different phytochemicals such as alkaloids, carbohydrates, phenolic compounds, glycosides, flavonoids, quinones, tannins, saponins, and triterpenoids were detected of the extracts of both medicinal plants. The outcomes of this investigation indicates that the extracts of *Hibiscus rosa sinensis* and *Nerium oleander* leaves may be the potential source of natural antioxidants that may be significant as medicinal agents in inhibiting or reducing the advancement of ageing and different neurodegenerative diseases. Additionally, it offers the foundation for focused chemical isolation and more accurate investigational work. The type of solvent employed has an important impact on how well a phytochemical can be extracted from plant material. Similar to this, the test used for phytochemical analysis establishes if a phytochemical is present or absent in the sample. As a result, various tests were run to produce accurate results for the detection of phytochemicals in the plants. Therefore, it is crucial to investigate its potential for new applications in the field of pharmaceutical and medical sciences. Phenol and flavonoids, two phytochemicals that function as antioxidants and aid in disease prevention. Natural antibiotics like saponins are produced. Since they have undergone rigorous efficacy testing and are largely considered as safe for use by humans, traditional and ethnobotanical uses of natural chemicals, particularly those with plant origins, have attracted a lot of attention recently. It is the most effective conventional strategy while looking for novel compounds to treat diverse ailments. AChE, BuChE, beta-amyloid, BACE 1, tau protein, MAO A, and NMDA are only a few of the several targets involved in the complicated neurodegenerative disease known as Alzheimer’s disease. To treat the symptoms and have effects that affect the course of the disease, several compounds have

been created against various targets. Despite the strenuous efforts, there are currently relatively few medications in the pipeline because of the molecule's inability to fulfil the ADME profiling. For these limitations, current study investigated the reprocessing of the existing approved antipsychotic drugs, which already have their pharmacokinetics, toxicological profiling, formulation development, and bulk production completed, saving money and time. According to computational approaches, *Hibiscus rosa-sinensis* and *Nerium oleander* Linn. are antipsychotics that are more effective at inhibiting the BuChE enzymes that causes Alzheimer's disease. The current analysis demonstrates that riboflavin and oleagenin are more potent inhibitors of human BuChE than Thioflavin T, a well-known inhibitor of BuChE, in relation to the binding energy values in our research investigation. To increase the effectiveness of these chemicals and learn more about the affecting pathways, cell and animal investigations are required. In silico medication repurposing has therefore proven successful in identifying promising outcomes that may be helpful therapeutically in Alzheimer’s disease.

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