CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Anticancer Evaluation of *Asarum canadense* Against Breast Cancer Using Computational and Invitro Approaches

by

Urooj Mumtaz

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

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

2024

I dedicate this thesis to my loving and supportive family and friends who have fully helped me in achieving my life goals.

CERTIFICATE OF APPROVAL

Anticancer Evaluation of *Asarum canadense* Against Breast Cancer Using Computational and Invitro Approaches

by

Urooj Mumtaz

(MBS223025)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Maria Shabbir	NUST Islamabad
(b)	Internal Examiner	Dr. Sohail Ahmad Jan	CUST Islamabad
(c)	Supervisor	Dr. Erum Dilshad	CUST Islamabad

Dr. Erum Dilshad
Thesis Supervisor
April, 2024

Dr. Syeda Marriam Bakhtiar
Head
Dept. of Bioinfo. and Biosciences
April, 2024

Dr. Sahar Fazal
Dean
Faculty of Health and Life Sciences
April, 2024

Author's Declaration

I, **Urooj Mumtaz** hereby state that my MS thesis titled "**Anticancer Evaluation of *Asarum canadense* Against Breast Cancer Using Computational and Invitro Approaches**" is my own work and has not been submitted previously by me for taking any degree from Capital University of Science and Technology, Islamabad or anywhere else in the country/abroad.

At any time if my statement is found to be incorrect even after my graduation, the University has the right to withdraw my MS Degree.

(Urooj Mumtaz)

Registration No: MBS223025

Plagiarism Undertaking

I solemnly declare that research work presented in this thesis titled "**Anticancer Evaluation of *Asarum canadense* Against Breast Cancer Using Computational and Invitro Approaches**" is solely my research work with no significant contribution from any other person. Small contribution/help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Capital University of Science and Technology towards plagiarism. Therefore, I as an author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of MS Degree, the University reserves the right to withdraw/revoke my MS degree and that HEC and the University have the right to publish my name on the HEC/University website on which names of students are placed who submitted plagiarized work.

(Urooj Mumtaz)

Registration No: MBS223025

Acknowledgement

All praise and thanks to the Supreme Allah to whom we only bow down. I would also like to express my gratitude to my family and friends for their continuous mental and physical support and prayers. I would also wholeheartedly say a big thank you to my supervisor Dr. Erum Dilshad (Assistant Professor, Department of Bioinformatics and Biosciences, CUST) for her support with that I would say a special thanks to Miss Iqra and Zulkiful for giving their precious time to assist with computational approaches and sample provision.

Thanks to all.

(Urooj Mumtaz)

Abstract

Cancer is defined as uncontrolled cell division and over expression of genes leading to metastasis. Breast cancer is one of the deadliest type of cancers. Its worldwide prevalence has been remarkably increased in developing countries. The therapy available is not in the access of common men and holds crucial side effects. One option could be the inculcation of CAM medicines derived from herbal sources. As they show minimum to none life threatening side effects. They had been used since ancient times as they hold anti-inflammatory, anti- microbial, anti- allergen, anti-malarial and anti- tumor activities. The extracts of *Asarum canadense* contain essential bioactive compounds including aristolochic acids, flavonoids, quinolines and essential oils. This study was conducted to determine the antitumor role of candidate plant against breast cancer using computational approaches followed by in vitro testing. Target proteins brcaa1, PR, ER and Ki-67 were docked against 10 bioactive compounds belonging to different classes using CB Dock2. The results indicated the need for further research on *Asarum canadense* as it provided significantly competent results as compared to gold standard of breast cancer drug, Tamoxifen. Organic extract showed less cell viability indicating high inhibitory activity as compared to aqeous extract against breast cancer cell lines. This opened doors to work on further FTIR analysis, cultivation and processing of plant to obtain its antitumor compounds. These compounds could then be used as alternative, cost effective and less side effects holding drug alternatives.

Keywords: Breast cancer, *Asarum canadense*, CB-dock2, ADMET, Tamoxifen, Ki-67, Estrogen Receptor, Progesterone Receptor

Contents

List of Figures

List of Tables

Abbreviations

A. canadense	*Asarum canadense*
ER	Estrogen Receptor
PR	Progesterone Receptor
PDB	Protein Data Bank
HER2	Human Epidermal Growth Factor Receptor 2
CAM	Complementary and Alternative Medicine
VDss	Volume of distribution at steady state

Chapter 1

Introduction

Cancer is defined as uncontrolled cell proliferation leading to an abnormal cell cycle. Breast cancer falls among one of the lethal and most commonly reported cancers. It is characterized by an uncontrollable character growth and division of abnormal breast cells that can permeate neighboring tissue causing spread to other parts of the body via process recognized as metastasis. It is treatable in 70–80% of patients with early-stage non-metastatic disease. With the current state of medicine, advanced breast cancer with metastases to other organs is thought to be incurable. Human epidermal growth factor receptor 2 activation, hormone receptor activation, and BRACA mutations are among the diverse molecular characteristics of breast cancer. Chemotherapy, hormone therapy, and the application of a bone-stabilizing medication are examples of systemic therapies, as well as immunotherapy. Future approaches to treating breast cancer focus on individualizing care and adjusting the level of treatment based on the biology of the disease and how it responds to therapy in the early stages. Another innovation in treatment requires the use of medicinal plants as inhibitors and anticancer agents [1].

Breast cancer is one of the most common cancers in women worldwide. Because breast cancer incorporates complex molecular mechanisms, prevention is better than treatment. Because of the complicated molecular variations, treating breast cancer with radiation treatment or chemotherapy therapy is challenging and often has unintended side effects. For centuries, plants and their extracts have been

utilized to address various ailments, including breast cancer. Herbal remedies are a reliable option for treating cancer due to their minimal toxicity. Additionally, women with breast cancer readily embrace herbal solutions due to their accessibility and affordability. In recent years, numerous plants and their compounds have displayed promising anti-cancer properties against breast cancer cells in both lab and animal studies. However, their efficacy in treating breast cancer remains uncertain due to the absence of randomized clinical trials [2].

Breast cancer exhibits a varied molecular profile. Activation of the human epidermal growth factor receptor 2 (HER2, encoded by ERBB2), activation of the progesterone and estrogen receptors, and/or BRCA mutations are among the molecular hallmarks of the illness. Depending on the molecular subtype, different treatment plans apply. Treatment for breast cancer is interdisciplinary and consists of both systemic therapy and a loco- regional strategy (surgery and radiation therapy). Hormone therapy for diseases that are hormone receptor positive, chemotherapy, anti-HER2 therapy for diseases that are HER2- positive, bone stabilizing medicines, poly (ADP-ribose) polymerase inhibitors for carriers of BRCA mutations, and, more recently, immunotherapy are examples of systemic therapies. Future approaches to treating breast cancer seek to reduce complications and strengthen treatment according to tumor biology and early response to treatment, while also tailoring therapy for each patient [3].

Breast cancer development frequently involves the activation of BRACA genes. They are important tumor suppressor genes, which suggests that through controlling cell division and mending broken DNA, they contribute in the prevention of cancer. The cells have capacity to correctly repair DNA may be compromised by mutations in BRACA1 and BRACA2, which increases the likelihood of mutations that could result in cancer. It is possible to inherit these gene mutations from one's parents. Those who carry these mutations are at an increased risk of developing metastases. By identifying these changes through genetic testing, people can assess their risk of developing cancer and decide on preventative and early detection measures like intensified surveillance, risk-reducing operations, or tailored medicines. Knowing the presence of BRACA mutations in breast cancer patients

can guide treatment decisions. Some treatments like PARP inhibitors, have been developed specifically for breast cancer with BRCA mutations [4][5].

Asarum canadense also termed as Canadian wild ginger is native plant to North America but also dispersed in South Asian countries. It contains various phytochemical compounds with potential biological activities. Some of these properties include; characteristic aroma and flavour due to the presence of volatile aromatic compounds such as asarone and methyl eugenol. It also contains certain alkaloids such as aristolochine and its derivatives. These alkaloids may have insecticidal and antimicrobial properties. Some species of wild ginger are known to contain flavonoids which are anti-oxidants and may hold various health benefits. Terpenoids are another class of compounds that are present in *Asarum canadense*. These compounds have diverse biological activities including anti-inflammatory and antimicrobial properties. Tannins are polyphenolic compounds that contain astringent properties. It also contains essential oils such as eugenol and cineole which contribute to its aromatic properties[6].

1.1 Problem Statement

Breast cancer, a leading cause of mortality worldwide, affects individuals of all genders. While conventional chemotherapeutic treatments are effective, they often entail significant side effects. Exploring alternative treatments, such as herbal or complementary medicine, presents an avenue for reducing adverse effects. In this study, we aim to evaluate the potential efficacy of bioactive compounds found in *Asarum canadense* against breast cancer through computational modeling and in vivo experiments.

1.2 Aim and Objectives of Study

The main aim of this investigation is to delve into any potential involvement of *Asarum canadense* in breast cancer therapy.

The **objectives** designed to achieve this aim of included:

1. To identify the potential bioactive compounds present in *Asarum canadense* with anticancer properties by computational approaches.
2. To investigate the interaction of bioactive compound of *Asarum canadense* with targeted protein of breast cancer by molecular docking.
3. To assess the cytotoxicity of *Asarum canadense* extracts against breast cancer cell lines in vitro.

1.3 Scope

Cancer is a disease that affects people all over the world. There is an increasing need for new therapies to treat and prevent this life-threatening disease. The research is focused on finding natural compounds because they are believed to have fewer side effects than existing treatments such as antibiotics. The plant kingdom has produced secondary metabolites that have been studied for their anti-inflammatory activities, leading to the development of new therapeutic agents. As these compounds are developed in cancer treatments, new technologies emerge that will further advance the field. New technologies include nanoparticles for nanomedicines, which aim to improve the anti-cancer properties of herbal medicines by controlling the release of compounds and exploring new delivery systems[7].

The present techniques for treating breast cancer are linked to possible catastrophic adverse effects. A more effective option that could treat patients in tandem while having little to no side effects is complementary and alternative medicine (CAM). Numerous wild plants scattered over the globe have been found to have bioactive chemicals that can prevent unregulated division of cells and proliferation. According to recent studies, such compounds are predicted to be found in *Asarum canadense* as well[6]. Therefore, there is a need to dock these bioactive compounds against proteins distinguishing in breast cancer patients so that

their inhibitory properties could be studied well coupled with their efficacy against anticancer therapy. In Silico molecular docking shall serve the purpose. In addition, invitro experimentation shall also be conducted to provide solid assistance to computational predictions.

Chapter 2

Literature Review

2.1 Cancer

Uncontrolled development and dissemination of aberrant cells throughout the body is the hallmark of cancer, a complex and multifaceted collection of disorders. It can start in almost any organ or tissue, spread to nearby tissues, or even spread to other regions of the body through metastases. Genetic mutations that accumulate over time and interfere with the regulatory systems that govern cell growth, division, and death are frequently the root cause of cancer formation. Because of this, cancer cells are able to develop their own blood supply, circumvent the body's defenses, and continue to grow and survive. Over time, our understanding of cancer has grown dramatically, resulting in more profound insights into its underlying molecular pathways, more effective targeted medicines, and better diagnostic tools [8].

2.1.1 Symptoms of Cancer

Cancer frequently manifests as unexplained weight loss, exhaustion, continuous discomfort, pale complexion, abnormal changes in bowel or bladder habits, persistent cough, difficulty swallowing, body lumps, abnormalities in moles or warts,

and spontaneous bleeding. Breast cancer symptoms include lumps in the breast or underarm, changes to the breast's size, shape, or appearance, redness combined with puckering or dimpling that alters the texture of the breast skin, inverted nipples, and swelling [9].

2.1.2 Types of Cancer

Cancer encompasses a wide array of types, each originating in specific tissues or organs, and often exhibiting distinct characteristics and behaviors. Some of the most common types of cancer include breast cancer, lung cancer, and colorectal cancer. Breast cancer typically starts in the breast tissue and can occur in both men and women, though it is more common in women. Lung cancer develops in the lung tissues and is strongly associated with tobacco smoke exposure. Colorectal cancer, which affects the colon or rectum, usually begins as a benign growth called a polyp that can eventually become cancerous. Other noteworthy types of cancer include prostate cancer, which affects the prostate gland in men, and skin cancer, with melanoma being one of its most aggressive forms. Pancreatic cancer originates in the pancreas and is often diagnosed at an advanced stage due to its asymptomatic nature in early stages. Additionally, leukemia affects the blood and bone marrow, causing abnormal white blood cell production. Ovarian cancer impacts the ovaries and is often diagnosed in later stages, contributing to its challenging prognosis[10].

2.2 Statistics

Among the leading causes of death worldwide is cancer. According to the Global Burden of Disease Cancer, there were about 17.5 million diagnoses of cancer in 2015, and 8.7 million deaths from the disease. Of all the cancers, breast cancer is one of the most serious global health issues. Breast cancer accounted for 13.8% of all cancer cases in 2012, making it the most frequent malignancy. According to a

2014 study done in China, India, and Russia, breast cancer was the second most common cause of death for women, behind lung cancer [4].

2.3 Breast Cancer

Breast cancer is a prevalent malignancy that affects both men and women globally. This occurs when aberrant cells in the breast tissue proliferate out of control, resulting in a tumor that has the ability to infiltrate neighboring tissue and maybe spread to other body areas. The milk ducts, also known as lobules, are the organs of the breast that produce and distribute milk. This kind of cancer frequently starts in these structures. Although the precise causes of breast cancer are still unknown, a number of risk factors have been found. Gender is significant because women have higher levels of estrogen and progesterone than men, which puts them at greater risk. Certain types of breast cancer cells may develop more quickly as a result of these hormones.

Age is another important factor as the risk of developing breast cancer increases with age. Family history and genetics also play a role; people whose close relatives have breast cancer are at increased risk of developing the disease, and some genetic mutations, such as mutations in the BRCA1 and BRCA2 genes, are associated with a significantly higher risk [4][11]. Early diagnosis is the key to improving the prognosis of breast cancer. A regular breast self-exam and a clinical breast exam by a healthcare professional can help detect any unusual changes in your breasts. Mammograms, or x-rays of breast tissue, are effective tools for early detection of breast cancer, even before physical symptoms appear [11].

2.4 Types of Breast Cancer

Breast cancer is not a single disease but encompasses several different types, each with distinct characteristics.

The two primary categories of breast cancer are invasive and noninvasive. Within these categories, there are various subtypes of breast cancer. Some of the most common types include:

- Ductal Carcinoma In Situ (DCIS): This is a non-invasive cancer where abnormal cells are found in the lining of a breast duct. It's considered the earliest form of breast cancer.
- Invasive Ductal Carcinoma (IDC): This is the most common type of invasive breast cancer. It begins in the milk ducts and then invades surrounding tissues in the breast.
- Invasive Lobular Carcinoma (ILC): ILC starts in the milk-producing glands (lobules) and can also spread to nearby tissues.
- Triple-Negative Breast Cancer: This is a subtype of breast cancer that lacks estrogen receptors (ER), progesterone receptors (PR), and HER2/neu receptors. It can be more challenging to treat.
- HER2Positive Breast Cancer: Some breast cancers overexpress the HER2/neu protein, making them more aggressive. Targeted therapies like Herceptin are used to treat this subtype.
- Luminal A and Luminal B: These subtypes are based on the presence of hormone receptors (ER and PR). Luminal A cancers are often low-grade, while Luminal B cancers tend to be more aggressive.
- Inflammatory Breast Cancer: This is a rare and aggressive type of breast cancer characterized by redness and swelling of the breast. It can often be mistaken for an infection.
- Phyllodes Tumours: These are rare tumors that develop in the stroma (connective tissue) of the breast.
- Male Breast Cancer: While breast cancer is more common in women, men can also develop breast cancer. It can be of various types similar to those in women.

- Metastatic Breast Cancer: This refers to breast cancer that has spread to other parts of the body, typically the bones, liver, lungs, or brain. It can be any subtype of breast cancer [5][12].

2.5 Available Treatment

A biopsy—a small sample of tissue taken from the suspected area and analyzed under a microscope—as well as imaging tests like mammography and ultrasound are necessary for the diagnosis of breast cancer. Following a diagnosis, breast cancer is categorized into several stages according to the tumor's size, extent, and whether or not it has spread to neighboring organs or lymph nodes. You can use this classification to guide treatment decisions. The kind, stage, and overall health of the patient are among the variables that affect the treatment options for breast cancer. Resection of the tumor and a small amount of surrounding tissue is known as a lumpectomy, and taking out the breast as a whole is known as a mastectomy. Surgery is a typical choice. After surgery, radiation therapy is frequently used for eliminating any cancer cells that survive by targeting them with high- energy radiation. Two systemic treatments that target cancer cells throughout the body include chemotherapy and targeted therapy [13].

Hormone therapy, particularly for hormone receptor-positive breast cancers, blocks hormones that fuel cancer growth. In recent years, targeted therapies have shown significant promise in treating certain types of breast cancer. HER2-positive breast cancers, for instance, overexpress the HER2 protein, which promotes cancer growth. Targeted therapies like Herceptin specifically target HER2-positive cells, improving treatment outcomes and reducing side effects [2].

Treatment for breast cancer is very individualized and is based on the patient's choices, general health, and the type and stage of her cancer. A variety of therapies are typically used in combination for treatment, including hormone therapy, radiation therapy, chemotherapy, targeted therapy, and surgery. Chemotherapy is a popular treatment for breast cancer that is frequently combined with radiation,

surgery, or other forms of treatment. To target and eradicate cancer cells, potent medications are used. Depending on a number of variables, including your overall health, the stage and type of your breast cancer, and your personalized treatment plan, your particular chemotherapy regimen may change. Chemotherapy medications for breast cancer often consist of cyclophosphamide, Texans (like paclitaxel), and anthracyclines (like doxorubicin). Treatment cycles may vary in duration and frequency and may cause side effects such as hair loss, nausea, fatigue and reduced blood cell counts [13][2]. Precision medicine has gained importance in recent years, tailoring treatments based on the individual genetic and molecular profiles of tumours. This approach is particularly effective in certain types of cancer, such as HER2-positive breast cancer, where targeted therapies specifically target the overactive HER2 protein. Immunotherapy, another innovative treatment, uses the immune system to recognize and attack cancer cells. As our knowledge of the genetic and molecular mechanisms of cancer increases, the development of personalized and targeted therapies offers hope for more effective and less invasive treatment strategies for various cancer types [2].

2.6 Medicinal Plants

Plant extracts, such as Taxol derived from the Pacific yew tree, have shown promise in treating breast cancer by disrupting cancer cell division [2]. However, rigorous clinical trials are needed to establish the safety and efficacy of botanical treatments. *Asarum canadense* is a medicinal plant with potential bioactive compounds [6].

2.7 *Asarum canadense*

Asarum canadense, (shown in Figure 2.1) also known as Canadian wild ginger, contains various bioactive compounds, including aaristolochic acids, asarone, volatile oils, tannins, and flavonoids. These compounds contribute to its potential medicinal properties and have been studied for their potential effects on health. Keep

in mind that the composition of bioactive compounds in plants can vary based on factors such as location, growing conditions, and extraction methods. *Asarum canadense* contains aristolochic acids, which have been studied for their potential anticancer properties. However, it's important to note that aristolochic acids have also been associated with toxic and carcinogenic effects, and their use is highly controversial due to these safety concerns. *Asarum canadense* primarily contains aristolochic acid I and aristolochic acid II, which are the two main types of aristolochic acids commonly found in various species of the aristolochic plant family, to which *Asarum canadense* belongs. These compounds have been the subject of research due to their potential medicinal properties as well as their associated health risks. Taxonomic classification of selected plant has been described in Table 2.1 [6].

FIGURE 2.1: Phenotype of *Asarum canadense*[14]

The flowers have a tubular or bowl shape and three small-tipped, dark crimson to brown triangular sepals that resemble petals. Flowers are two inches wide from tip to tip, and tubes range in diameter from 3/4 to 1 inch. The tube's interior is a milky white color, with a ring of twelve stamens in the center around by six

reddish-brown ones. Long white hairs cover the outside of the tube, especially in the area close to the base. There is only one blossom on the plant, and it is located in the earth near its base. Fruit is a capsule that holds several seeds. Wild ginger spreads vegetatively from rhizomes and typically grows in clusters. The color of the blooms in the springtime is readily obscured by the dark leaves of the woodland., but the leaves can be easily identified. It makes an excellent ground cover for a shady garden. It has nothing to do with the ginger plant popular in Asian cuisine [6][14].

TABLE 2.1: Taxonomic hierarchy of *Asarum canadense* [14]

Kingdom	**Plantae**
Phylum	Angiosperms
Class	Eudicots
Order	Piperales
Family	Aristolochiaceae
Genus	Asarum
Species	*Asarum canadense*

Perennial herbs growing horizontally on the ground, with green, heart-shaped leaves, purple-brown cup-shaped leaves, three-parted sepals, and one flower per plant. There are sixty different perennial woodland species of the genus Asarum. Its leaves are soft, kidney shaped, long-long and show a special iridescent colour in all sunlight. The underground buds are shallow-growing, fleshy rhizomes that stand out to form clusters. The flowers bloom from April to June. The flowers are hairy and have three petals[14].

Chuanxiong and Asarum possess analgesic, anti-inflammatory, cardiovascular, and anti-cancer properties due to their volatile oil content. Traditional prescriptions, like Denxiang Chatiaosan and Denxiong Shinshinyu, combine these herbs for enhanced therapeutic effects [15].

2.8 Proteins Found in Breast Cancer Cells

Breast cancer cells can contain a wide range of proteins, and the specific proteins found in individual people can vary depending on the type of breast cancer.

According to immune-histochemical studies and molecular tests, some proteins commonly associated with breast cancer are human epidermal growth receptor 2 (HER2), estrogen receptor (ER) and progesterone receptor (PR), BRACA1 and BRACA2, Ki-67, p53, cyclin D1, Bcl -2 and epidermal growth factor receptor (EGFR)[16]. Table 1.2 indicates the mutated genes in breast cancer and their reported inhibitors.

TABLE 2.2: Mutated genes in cancerous cells and their respective reported inhibitors[16][17]

MUTANT	**INHIBITOR**
HER2	Trastuzumab (Herceptin)
	Pertuzumab (Perjeta, monoclonal antibodies)
ER	Tamoxifen
	Fulvestrant
PR	No possible inhibitors
BRCA1	
BRCA2	PARP (Poly ADP-ribose Polymerase) inhibitors:
	Olaparib, Rucaparib
Ki-67	No specific Inhibitors known
P53	Nutlin-3
Cyclin D1	CDK4/6 Inhibitors:
	Palbociclib, Ribociclib, Abemaciclib

2.9 Inhibitors Present in *Asarum canadense*

Asarum canadense contains certain chemical entities among which few are known. They include essential oils, flavonoids, aristolochic acid and tannins.

Some flavonoids such as quercetin, epigallocatechin have shown potential anti-cancer properties in laboratory and animal studies[17].

2.10 Molecular Docking

Molecular docking has been in use for past three decades for designing the drug through computer assistance and to find different structures in molecular biology.

Docking is preferred while performing virtual screening on the compounds present in the databases or libraries for analysis of their functions, results can be classified easily through docking and one of the main roles played by docking is to give the analysis of how the ligand interacted with the protein, locking it for optimizing the lead compounds for drug development[18].

Different docking programs uses either one or more search algorithms for the prediction of possible results of the receptor-ligand complex. This is the core reason for molecular docking to become a key tool for drug discovery and for molecular modelling applications. The docking result gives a score of the interaction and the accuracy of the scoring function makes docking more reliable for predicting the ligand pose and through that the binding site of the ligand can also be determined. With this it predicts the binding affiliation which in turn leads to the identification of a potential lead drug in association with the target protein[19].

Chapter 3

Materials and Methods

3.1 Selection of Disease

Breast cancer, a multifaceted disease, encompasses various molecular characteristics such as human epidermal growth factor receptor 2 (HER2, encoded by ERBB2), hormone receptor activation (progesterone and estrogen receptors), and /or BRCA mutations. These molecular subtypes dictate diverse treatment approaches. The selection of breast cancer as the focus of this study was motivated by its increasing prevalence in South Asian communities [3]. Understanding the molecular intricacies of breast cancer is paramount for developing targeted therapies tailored to individual patients' needs.

3.2 Selection of Proteins

In the context of breast cancer, numerous proteins with reported mutations were meticulously chosen for further investigation and analysis. Notable examples include BRACA1, ER, PR, and Ki67 [16][17]. The selection criteria were based on existing literature reports documenting their involvement in breast cancer pathology. These proteins play crucial roles in cell growth regulation, hormone signaling, and proliferation, making them key targets for therapeutic interventions. By

comprehensively analyzing the molecular alterations in these proteins, we aim to uncover potential biomarkers and therapeutic targets for breast cancer management.

3.3 Determination of Physicochemical Properties of Proteins

Using computational tools, the physicochemical properties of selected properties were noted to make further analysis and evaluation smooth. As physicochemical properties help to predict medication type. ExPASy: ProtParam tool was used which calculated the physicochemical parameters of proteins. This tool calculated both pI and mW as well as predicted AA composition, atomic composition, extinction coefficient, estimated half-life, and volatility index among others[20].

3.4 Cleaning of the Downloaded Protein

Pymol, an open-source gadgets, was used to extract the additional components that were associated to the protein after the protein structure was downloaded. To enable efficient completion of subsequent steps, the protein's remaining components will be removed, leaving only the linear chain, known as the A chain, which is composed of amino acids 1- 306 [21].

3.5 Determination of Functional Domains of Target Proteins

For determining the domains of the target protein InterPro, a database that can analyze a protein was used so that it also provided information regarding the families, functional sites and the domains of the proteins under study[22]. By

inserting the FASTA sequence of the main protein the polypeptide binding sites and homodimer interfaces were known.

3.6 Selection of Active Metabolic Ligands

In our pursuit of identifying potential therapeutic agents, computational tools played a pivotal role in the selection process. Active ligands were chosen based on reported data showcasing antiviral and antioxidant properties. Leveraging the extensive database of DrugBank, we retrieved bioactive compounds with established efficacy. Among the selected ligands are terpenes, quercetin, myrcene, neral, asarinin, and borneol derivatives [23]. These compounds exhibit diverse pharmacological activities and hold promise as candidates for further evaluation in our study.

3.7 Ligand Preparation

The preparation of ligands for molecular docking studies involved several steps to ensure accuracy and reliability. Initially, the 3-dimensional structures of the selected ligands were obtained from the PubChem database, a valuable resource managed by the National Center for Biotechnology Information (NCBI). PubChem provides comprehensive information on chemical molecules, including their names, molecular formulas, structural representations, and biological activities [22][24].

Upon acquiring the ligand structures, energy minimization was performed using Chem3D Ultra to optimize their molecular conformations. This step is crucial for ensuring that the ligands adopt energetically favorable configurations for subsequent interactions with target proteins. Energy minimization was repeated thrice to refine the ligand structures and enhance their stability.

In cases where the 3D structure of a selected ligand was unavailable, canonical smiles from PubChem were utilized as an alternative. These canonical smiles were

imported into ChemDraw software to generate 3D representations, which were then subjected to energy minimization using Chem3D Ultra.

Finally, the energy-minimized structures of the ligands were saved in the sdf format, ready for subsequent molecular docking simulations. This meticulous preparation process ensures the reliability and accuracy of ligand conformations, laying the foundation for meaningful interactions with target proteins.

3.8 Molecular Docking

For performing the molecular docking between the protein and the ligand, CB-dock 2 (Cavity detection guided blind docking) updated version was used. CB dock2 finds the sites of docking automatically. CB-Dock2 is a tool used for protein and ligand docking which indicates about the sites of bonding, the size and the centre is calculated. The box size was adjusted according to the ligand and then docking was performed. The docking was performed through Autodocking based on Vina score. As such docking is focused on cavity binding so ratio of accuracy is higher[25]. For performing the docking, we uploaded the 3D structure of protein in pdb format and the 3D structure of ligand in the sdf format. After this docking was run. The end result would be 5 different poses of interaction. To select the best pose, we opt for the minimum vina score which is given in KJ/m-1. CB-Dock will provide an interactive 3D visualization of results in 5 different poses. Best pose was selected on basis of minimum vina score given in (kJ/m-1)[26][27].

3.9 Visualization of Docking Result Via Pymol

Over the past few years, the PyMol has emerged as an efficient molecular tool of visualization. The graphics and its ability to view 3D structures have been extraordinary[27]. PyMol provides a plugin which can access the results and make their visualization clearer so that the docking results can be easily studied. The

pictures of the docking result can be captured also[28]. For all the process the docking result were saved in the pdb format and after visualization in the PyMol were also saved in the pdb file format.

3.10 Analysis of Docked Complexes via Ligplot

Once retrieved the docked complex with the lowest vina score, the lead to the analysis of the complex. The complex was in the pdb format. This analysis was done by using the software LigPlot. For the given pdb file format the schematic diagrams of the protein and ligand interactions were generated automatically. These interactions were modified by hydrogen bonds and through hydrophobic contacts. LigPlot provided the analysis of the hydrophobic and hydrogen bonding interactions. With this LigPlot generates the 2D representation of the protein-ligand complex[29][30].

3.11 Ligand ADMET Properties

After the analysis the study of pharmacokinetic and toxicity properties was done. The weak candidates of the drug were eliminated during preclinical ADME. The remaining candidates were selected as potential drugs against the disease. By using the PkCSM optimization of the ADME which is Absorption, Distribution, Metabolism and excretion related to human body was done[31].

3.12 Lead Compound Identification

After all the tasks were performed the lead compound was identified. The lead compound was identified after applying the rule of 5 which include:

1. The log value of the drug-like compound must be limited to 5.

2. The molecular weight should also be lesser than 500.

3. Hydrogen bond acceptors maximum number should be 10.

4. Hydrogen bond donors' maximum number should be 5[32].

The compound fulfilling screening of docking score, ADMET properties coupled with Lipinski's rules of five, was selected as our lead compound.

3.13 Comparison with Standard Drug

Tamoxifen drug which has shown antitumor properties against Breast cancer caused due to ER, PR mutations, has been selected as a standard drug for comparison against the lead compound. Olaparib drug could be selected in case of breast cancer developed due to mutations in BRACA1 genes[33].

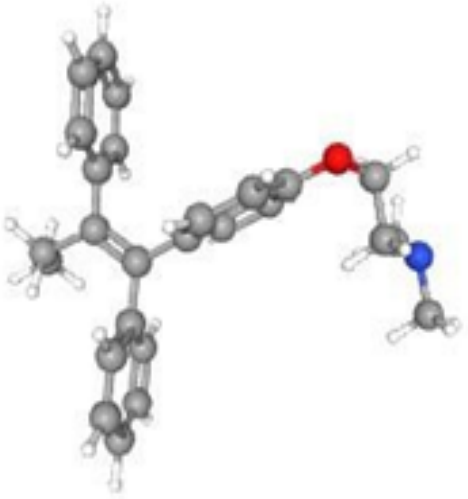


FIGURE 3.1: 3D structure of tamoxifen

Tamoxifen (brand names: Nolvadex, Saltamox) is a selective estrogen receptor (recurrence) modulator used in early recurrence (recurrence) of breast cancer in women or men after surgery to reduce the risk of hormone receptor positivity. SERM. For the treatment of advanced stage hormone receptor positive breast cancer in women or men. To reduce the risk of invasive breast cancer in women

diagnosed with hormone receptor-positive DCIS (ductal carcinoma in situ) and to reduce the risk of breast cancer in women with above-average risk of undiagnosed disease after surgery[34]. Physicochemical properties and molecular binding results determine drug candidate decisions.

Tamoxifen requires activation of cytochrome P450 (CYP) enzymes to produce the metabolites 4-hydroxytamoxifen and enddoxifen. Both metabolites have approximately 100 times greater affinity for the estrogen receptor than the parent drug and can inhibit cell proliferation. Polymorphic CYP2D6 is an important enzyme in this biotransformation, and recent mechanistic, pharmacological, and clinical evidence suggests that genetic alterations and drugs associated with CYP2D6 inhibitors affect plasma concentrations of active tamoxifen, its metabolites, and tamoxifen metabolites. In particular, inactive (poor metabolizers) and poor (moderate metabolizers) CYP2D6 alleles are associated with higher relapse rates [35].

3.14 Methodology for In Vitro Examination

To assess the potential impact of *Asarum* extract on breast cancer cells *in vitro*, a comprehensive methodology is employed. Breast cancer cells are initially cultured as controls, ensuring consistency. The *Asarum canadensis* extract is prepared meticulously, considering solvent selection and concentrations.

Subsequent experiments involve treating breast cancer cells with varying extract concentrations over time. Cytotoxicity and growth rate are assessed using established assays like MTT tests. Flow cytometry and Western blotting are utilized to explore cellular function, apoptosis induction, and protein expression changes associated with critical pathways.

Controls, including untreated cells and chemotherapy-treated cells, provide benchmarks for comparative analysis. This systematic approach integrates cell culture techniques with molecular and biochemical analyses to elucidate the extract's anticancer potential *in vitro* [20].

3.14.1 Sample Preparation

Asarum canadense samples were collected from Hurnamaira, district Rawalakot Azad Kashmir. Aerial parts of plant were taken and wrapped in towel paper. Leaves were air dried and crushed using pestle and mortar into fine powdered form. Two solvents were used; one to prepare aqueous solvent extract: water would be added by taking 100g dried sample in 500ml of double distilled water and heated using water bath followed by storage. Methanolic solvent extract was prepared by adding 100 g of dried sample in 500ml of methanol, soaked for 3 days and stored in falcon tube. Labelling was done.

3.14.2 Selection of Breast Cancer Cell Lines

MCF-7 (ATCC-HTB-22) was used as breast cancer cell line due to its easy availability and accuracy [36].

3.14.3 MTT Assay Analysis

The MTT assay involves the conversion of the water-soluble yellow dye MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to insoluble purple formazan by the action of mitochondrial reductase. Then, the formazan is dissolved and its concentration is determined by optical density at a wavelength of 570 nm[33].

3.14.4 MTT Protocol

The protocol used was dependent on four major steps namely; Seed cells preparation, Addition of MTT, Solublization and Read out step. 96-well plate was used to seed 3000 to 5000 cells per well. 6 to 8 replicates were taken foe each condition. Positive control was used. Seeding of cells means attachment of cells to well which takes approximately 24 hours before treatment and MTT addition.

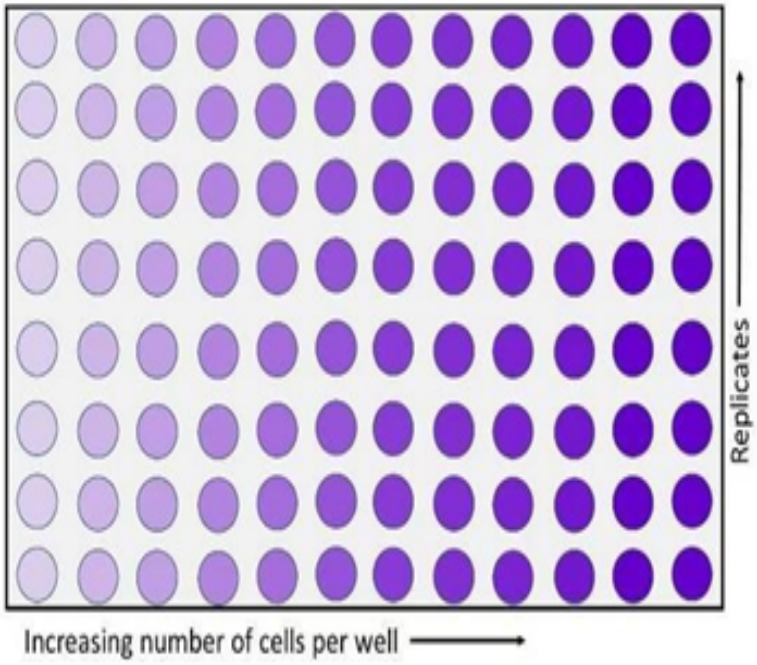


FIGURE 3.2: Conversion of MTT to farmazan in 96 wells

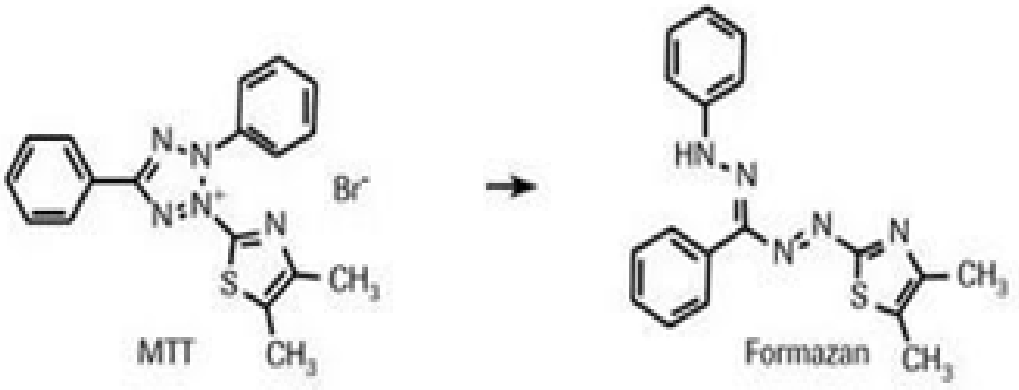


FIGURE 3.3: Conversion of MTT to formazan color switch from yellow to purple

5mg/mL MTT stock was made in Dpbs to save. From this, dillutions were made as 1:1000 in culture medium to get 5 micro gram per ml. 110 micro gram MTT media solution was added to each well. Each well should contain same number of cells for optimized and reliable results. The wells were inoculated for 4-6 hours at 37 degrees. 5%v CO2 was provided. After that media was removed carefully with a pipette without disturbing crystals. Pipette was washed everytime it removed media. 100 micro litres of DMSO per well was added and pipetted well to properly mix. Afterwards the samples were incubated for 10 minutes – 24 hours at Room

temperature. Microplate reader was used to read out the results at an absorbance of 550-600nm [37].

Two samples i.e., aqueous and organic nature were prepared. Cell lines were revived in optimum conditions, by maintaining aseptic conditions. Administration of different concentrations i.e., 250mm, 500mm and 1000mm of plant extract to cell lines was done. Cell viability was measured using MTT. Extracts toxicity to cell lines were determined. Assessment was done to check if the plant extract induces programmed cell death in tumor cells. Molecular mechanisms underlying the extracts was investigated. Data was analyzed using SPSS[38] [32].

Drug candidate will be suggested against breast cancer if it showed reasonable inhibitory activity or low cell viability.

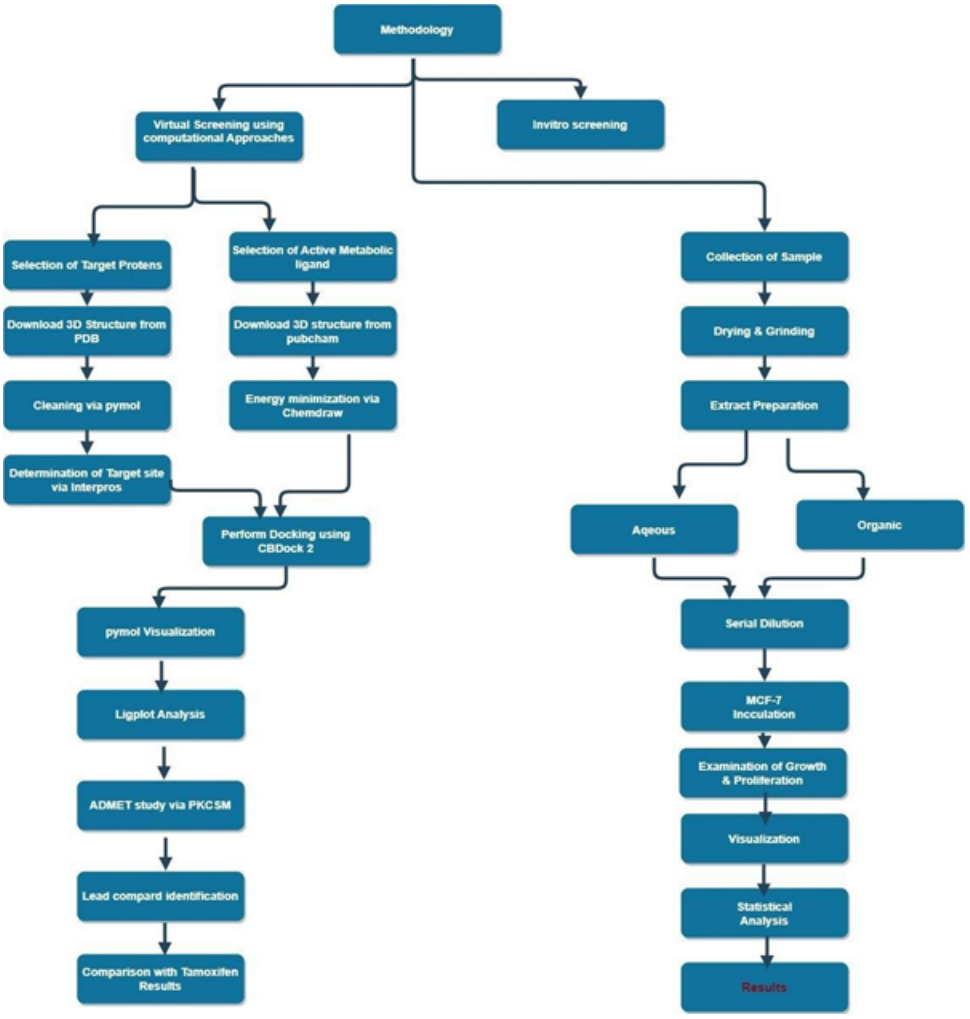


FIGURE 3.4: Overview of Methodology used for computational and In-vitro analysis

Chapter 4

Results and discussions

4.1 Structure Modelling

Ki-67, PR (progesterone receptor), and ER (estrogen receptor) were selected as targeted proteins against essential bioactive components present in *Asarum canadense*. These proteins act as molecular markers that play an important role in the prediction of responses to treatment and the overall prognosis. There elevation helped in proper diagnosis, therefore leading to appropriate therapy [1].

4.2 3D Structure of Proteins

Ki-67, ER and PR are all important markers used to understand the characteristics of cancer cells. Ki-67 helps measure the growth rate, while ER and PR indicate the presence of hormone receptors.

The 3D structures of these proteins were sourced from the Protein Data Bank (PDB) and downloaded. To prepare them for analysis, solvent and water molecules were removed using Pymol. Subsequently, the cleaned structures were saved in PDB format for further visualization and analysis using Pymol. This step ensures

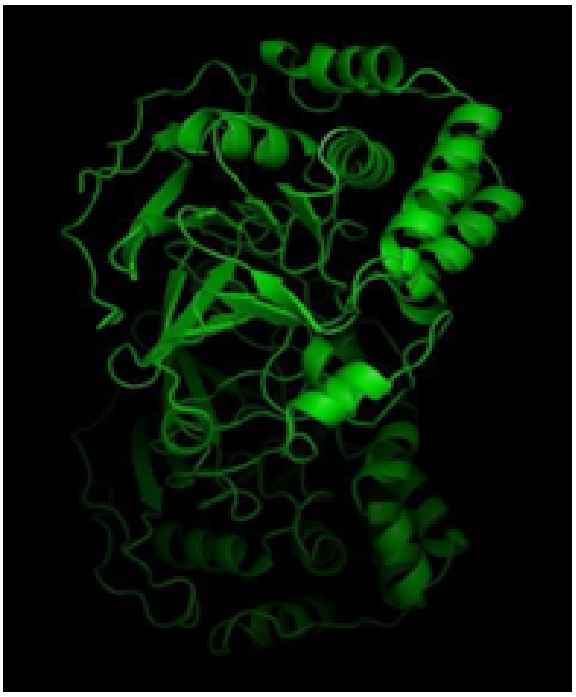


FIGURE 4.1: 3D visualization of Ki-67 protein

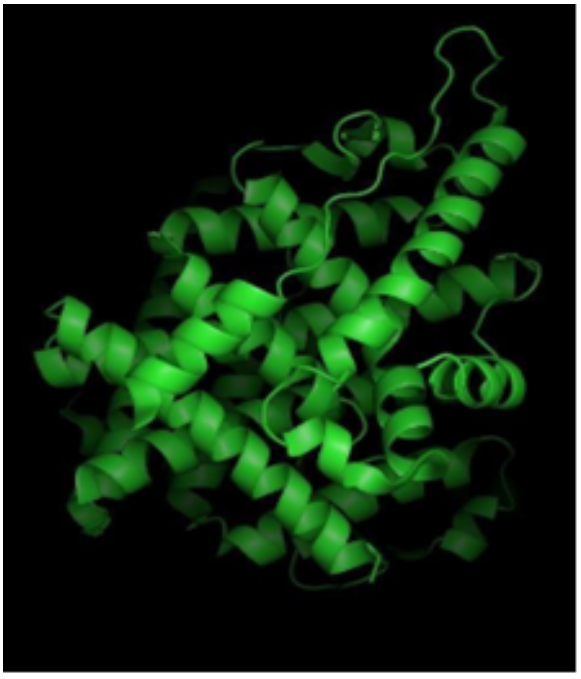


FIGURE 4.2: 3D visualization of ER protein

a clear understanding of the proteins' spatial arrangements, facilitating subsequent investigations into their functional roles in breast cancer.

Figures 4.1,4.2,4.3 shows the refined structure of selected protein using Pymol. Extra water molecules had been removed.

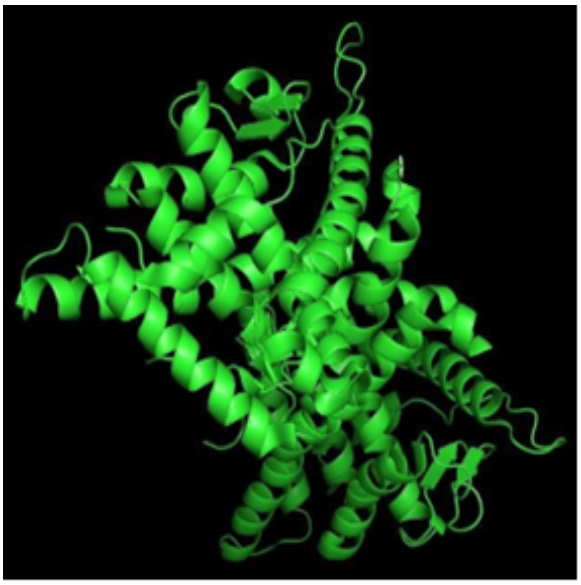


FIGURE 4.3: 3D visualization of PR protein

TABLE 4.1: Physicochemical properties of Target Proteins

Protein	**Ki-67**	**PR**	**ER**	**Brcaa1**
Accession No	P36873	P06401	P03372	Q3le39
Molecular weight	36983.79	98981.14	66216	147809.49
Pi	6.12	6.09	8.30	5.04
NR	44	90	60	273
PR	41	83	64	211
Ex.Co1	35130	68770	62520	89365
Ex.Co2	34380	67270	61770	88240
II	43.49	64.44	45.88	68.61
AI	89.97	81.76	80.39	61.95
GRAVY	-0.250	-0.177	-0.354	-1.093

4.3 Physical Properties of Protein

For studying the properties of our selected proteins tool of ExPASy called ProtoParam is used. It is an online tool that is used for computing the physical and chemical properties of proteins that are entered in Swiss-prot or TrEMBL or the proteins entered by the users. The parameters which are studied include molecular weight, proteins amino acid composition, atomic composition, estimated half life, extinction co efficient, instability index, aliphatic index and grand average of hydropathicity (GRAVY) [39].

Table 4.2 indicate essential properties of target proteins. It shows the molecular weight of proteins and their Ph. Pi values indicate that except ER are acidic in nature. The values of light absorption in terms of extinction coefficient are 35130,34380 for Ki-67, 68770,62720 for PR, 62520, 61770 for ER and 89365, 88240 for brcaa1 protein. Stability index value show that Ki-67 is most stable among others. Thermal index show that selected proteins fall in Thermostable range. Low values of GRAAVY show that Brcaa1 has good interactions with water molecules comparatively.

4.4 Ligand Selection

Ligands were selected on the basis of their physicochemical properties linked with already reported data. Many of the bioactive compounds has already been reported for their medicinal application and to their adverse effects, Random selection was done to pick 10 ligands that were more commonly occurring in other medicinal plants as well. Their hydrophilic and hydrophobic attractions made them more likely to hold clinical significance.

Table **??** indicates essential bioactive compounds present in plant candidate and their physical properties including their molecular weight, structure and chemical composition.

4.4.1 Virtual Screening of Selected Ligands using Lipinski Rule of five

For compounds to be dealt as drug like and non-drug like Lipinski rule of five and ADMET properties are considered vital. The Lipinski rule deals with certain parameters like Molecular weight which should be less than or equal to 500, log P less than or equal to 5, H-bond donors less than or equal to 5, H-bond acceptors less than or equal to 10. These rules are to be followed by orally active compounds. The drug like compounds are dependent on the mode of administration. Compound

TABLE 4.2: Physicochemical properties of Target Proteins

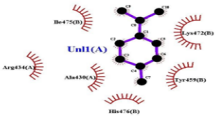
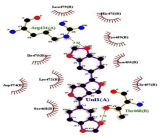
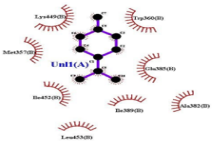
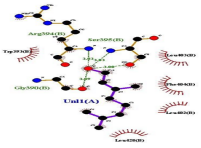
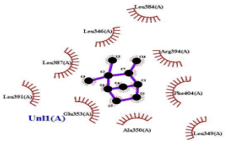
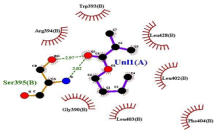
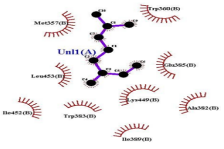
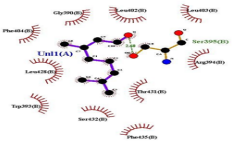
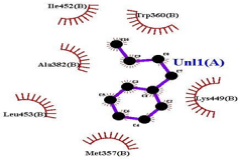
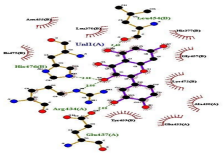
S. No	Ligand Name	Molecular Formula	Molecular Weight	Structure
1.	Borneol	C10H180	154.253	
2.	p-cymene	C10H14	134.222	
3.	Camphene	C10H16	179.32	
4.	Quercitin	C15H10O7	179.32	
5.	Isobutyl venylacetate	C11H14O2	142.198	
6.	β-phallandrene	C10H16	136.238	
7.	Neral	C10H16O	152.237	
8.	Myrcene	C10H16	136.238	
9.	α-terpinene	C10H16	136.238	
10.	Asarinin	C20H18O6	354.358	

TABLE 4.3: Applicability of Lipinski Rule on selected Ligands

Ligand	**Log P-Value**	**Molecular Weight(g/mol)**	**H-Bond Acceptor**	**H-Bond Donor**
Borneol	2.6698	154.253	1	0
P-cymene	3.0292	134.222	0	0
Camphene	2.9987	179.32	0	0
Quercitin	1.988	179.32	0	0
Isobutyl vinylacetate	1.9058	142.198	2	0
β-phallandrene	3.164	136.238	0	0
Neral	2.878	152.237	1	0
Myrcene	3.475	136.238	0	0
A-terpinene	3.3089	136.238	0	0
Asarinin	3.2192	354.358	6	0

to be regarded as Drug like should follow 3 or more rules and if any compound violates two or more rules is considered as least absorbed [35].

The table 4.3 shows that all ligands follow Lipinski rule.

4.4.2 Toxicity Prediction of Ligands

pkCSM is an online tool applied to predict ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) values of desired bioactive compounds and drugs. This tool was used to determine the above properties especially toxicity to better understand our compound. The AMES toxicity test uses bacteria to test the mutagenic potential of compounds. If a positive reaction is observed, the ligand is mutagenic and may act as a carcinogen[34][40].

T. piriformis toxicity method uses piriformis protozoan bacteria toxicity as the toxicity endpoint. Any value above $\geq$ 0.5 log μg/L is considered toxic. In the minnow toxicity test, the estimated value is used to represent the concentration of the compound that causes 50% death of minnows. Values below 0.5 mM are considered acutely toxic. The MRTD value indicates the starting dose of a drug in phase I clinical trials. Values $\leq$ 0.477 log mg/kg/day are considered low; values above are considered high. For oral toxicity studies in rats, the estimated log value of the least observed adverse effect is given as a function of the compound

concentration required for the duration of treatment. Hepatotoxicity test predicts whether liver function will be affected or not. A skin test predicts whether a skin reaction will occur. hERG I and II inhibition assays determine the ability of each compound to inhibit hERG-related potassium channels. Blockers of this channel will cause QT syndrome, and in the long term, the patient will develop ventricular arrhythmias [40].

4.4.2.1 Borneol, p-cymene, Camphene, Quercitin and Isobutylvinyl acetate

The toxicity values of above mentioned ligands are given below. Table 4.4 shows that Quercitin has high MRTD value. All other values for Quercitin and Camphene are in safe range that show these ligands are not the cause of AMES toxicity except Borneol, Pcymene and Isobutyl vinylacetate that show skin sensation Positive results. None of the ligands was found to be hERG I and II inhibitors. These ligands have safe toxic rates with respect to tests on T.pyriformis and tests on rats. However, Quercitin falls in slight toxic range. The lead compound till now is Camphene.

4.4.2.2 β-phallandrene, Neral, Myrcene, α-terpinene, and Asarinin

Table 4.5 presents toxicity values for the aforementioned ligands. Except for Asarinin, all other compounds fall within a safe range with respect to AMES toxicity values. Among these ligands, Neral exhibits the highest Maximum Tolerated Dose (MTRD) value. None of the mentioned ligands function as HERG I and II inhibitors. Additionally, all ligands demonstrate safety in toxicity tests on rats, with no observed liver toxicity. However, skin irritation is reported for β-phallandrene and Neral. In T.pyriformis tests, all ligands are classified within the safe zone. Both Myrcene and α-terpinene emerge as lead compounds. Final scoring will be based on the Absorption, Distribution, Metabolism, and Excretion (ADME) scores of these ligands.

TABLE 4.4: Comparison of Toxicity properties of Ligands

Traits	**Borneol**	**Pcymene**	**Camphene**	**Quercitin**	**Isobutyl-vinyl-acetate**
AMES Toxicity	No	No	No	No	No
Maximum tolerated dose	0.778	0.954	0.305	1.159	0.911
HERG-I inhibitor	No	No	No	No	No
HERG-II inhibitor	No	No	No	No	No
Oral Rat Acute Toxicity	1.693	1.781	1.554	1.944	1.993
Oral Rat Chronic Toxicity	2.046	2.294	2.247	3.169	2.371
Hepatotoxicity	No	No	No	No	No
Skin sensation	Yes	Yes	No	No	Yes
T-pyriformis toxicity	0.633	0.62	0.533	0.294	0.075
Minnow toxicity	1.095	0.918	1.19	1.305	1.481

4.5 Molecular Docking Results

Molecular docking is a technique applied for the estimation of linkage strength between a ligand bonded to a receptor protein through vina score function and for determining the correct structure of Ligand that binds to the binding site.3D structures of Ligand and Receptor are pre requisites for docking. After obtaining the required structures an online freely available tool CB Dock 2 was used. CB Dock2 predicts the binding sites of protein and calculates the cavity sizes. After Docking, CB Dock gives us the five best poses and receptor models. Among these five models best pose was selected on the basis of vina score and size of cavity[41][42].

TABLE 4.5: Comparison of Toxicity Properties of Ligands

Traits	**βphallan -drene**	**Neral**	**Myrcene**	**αterpinene**	**Asarinin**
AMES Toxicity	No	No	No	No	Yes
Maximum tolerated dose	0.754	0.866	0.67	0.693	0.346
HERG-I inhibitor	No	No	No	No	No
HERG-II inhibitor	No	No	No	No	No
Oral Rat Acute Toxicity	1.741	1.921	1.683	1.744	2.876
Oral Rat Chronic Toxicity	2.328	2.0288	2.415	2.369	1.619
Hepatotoxicity	No	No	No	No	No
Skin sensation	Yes	Yes	No	No	No
T-pyriformis toxicity	0.638	1.038	0.925	0.561	0.321
Minnow toxicity	0.942	1.107	0.646	0.963	0.321

Molecular docking was performed using four different proteins that were selected including brcaa1, Ki-67, ER and PR separately. 10 ligands were selected as mentioned above in table no 4.2. 3D proteins structures were downloaded from PDB. They were cleaned using Pymol removing water molecules and saved in PDB format. 3D structure of Ligands was obtained using PubChem in sdf file format. Energy minimization of ligand was done using Chemdraw and saved as sdf file. CB Dock 2 was used for docking. Both the structures were uploaded and docking was run. Docking results in complexes formation showing various hydrogen bonds between ligand and receptor protein molecules[43].

Table 4.6 shows the docking results of five selected ligands that are Borneol, p-cymene, Camphene, Quercitin and Isobutylvinyl acetate. It shows that Borneol has binding score of -6.0 which is highe than that of Camphene, Quercitin and Isobutyl vinyl acetate but lower than p-cymene binding score that is -5.9. The log p value for this docking is 2.6.

TABLE 4.6: Results of Docking Ligands 1-5 with PR (Progesterone Receptor)

Compound	**Borneol**	**p-cymene**	**Camphene**	**Quercitin**	**Isobutyl vinyl-acetate**
Binding score	-6.0	-5.9	-6.4	-8.3	-6.1
Cavity Volume	778	778	778	778	778
Molecular weight	154.253	134.222	179.32	179.32	142.198
Log P	2.6	3.0	2.9987	1.988	1.9

TABLE 4.7: Docking Results of Ligands 6-10 with PR

Compound	**β-phallan-drene**	**Neral**	**Myrcene**	**α-terpinene**	**Asarinin**
Binding score	-4.6	-4.6	-5.6	-6.0	-10.3
Cavity Volume	778	778	778	778	778
Molecular weight	136.238	152.237	136.238	136.238	354.358
Log P	3.1	2.8	3.4	3.3	3.2

TABLE 4.8: Results of docking Ligands 1-5 with ER (Estrogen Receptor)

Compound	**Borneol**	**p-cymene**	**Camphene**	**Quercitin**	**Isobutyl vinyl-acetate**
Binding score	-5.5	-5.8	-5.8	-8.8	-6.8
Cavity Vol.	4245	4245	4245	4245	4245
Molecular weight	154.253	134.222	179.32	179.32	142.198
Log P	2.6	3.0	2.9987	1.988	1.9

Above table 4.7 shows the binding scores for remaining ligands. Asarinin shows -10.3 binding score that is highest as compare to others. Binding score trend is as:

Asarinin > **α-terpinene** > **Myrcene** > **Neral and β-phallandrene**

P log values indicate that Neral has lowest value of 2.8 value and Myrcene has 3.4 highest among others.

TABLE 4.9: Results of Docking Ligands 6-10 with ER (Estrogen Receptor)

Compound	**β-phallan -drene**	**Neral**	**Myrcene**	**α-terpinene**	**Asarinin**
Binding score	-6.0	-5.7	-5.7	-5.9	-9.7
Cavity Vol.	4245	4245	4245	4245	4245
Molecular weight	136.238	152.237	136.238	136.238	354.358
Log P	3.1	2.8	3.4	3.3	3.2

TABLE 4.10: Results of docking Ligands 1-5 with Ki-67

Compound	**Borneol**	**p-cymene**	**Camphene**	**Quercitin**	**Isobutyl vinyl-acetate**
Binding score		-5.6	-5.6	-8.6	-4.6
Cavity Vol.		2300	550	300	322
Molecular weight	154.253	134.222	179.32	179.32	142.198
Log P	2.6698	3.0292	2.9987	1.988	1.9058

Table 4.8 shows the binding scores of selected ligands against Protein 2 that was ER protein. It shows the binding score trend as:

Quercitin > Camphene and p-cymen > Borneol

p-cymene shows highest log value of 3.0 among others.

Table 4.9 shows that Asarinin holds highest binding score of -9.7 as compare to other ligands under discussion. Binding score trend is as:

Asarinin > β-phallandrene > α-terpinene > Neral = Myrcene

Myrcene shows highest log P value about 3.4 and β-phallandrene shows least log P value of about 3.1.

Table 4.10 displays the binding affinities of selected ligands with our target proteins. Quercetin exhibits the highest binding score of -8.6. Interestingly, the binding score suggests that p-cymene and Camphene share an equal affinity to bind with the Ki-67 protein.

TABLE 4.11: Results of Ligands 6-10 with Ki-67

Compound	**β-phallandrene**	**Neral**	**Myrcene**	**α-terpinene**	**Asarinin**
Binding score	-5.6	-5.2	-5.3	-5.6	-9.1
Cavity Vol.	N/A	550	2300	2300	2300
Molecular weight	136.238	152.237	136.238	136.238	352.358
Log P	3.164	2.878	3.475	3.3089	3.2192

Table 4.11 shows that Asarinin holds maximum binding affinity with Ki-67 bearing binding score of -9.1. β-phallandrene and α-terpinene shared binding affinity by getting -5.6 binding score.

4.6 Interaction of Ligands and Targeted Proteins

The results deduced from Docking were analyzed using Pymol and Ligplot. The interaction between the Ligands and receptor Protein was predicted through Ligplot plus. The graphical system of LigPlot automatically generated 2D pictures of interactions from its 3D coordinates. The dotted line indicates the presence of Hydrogen bonds. 2D picture exhibits not only hydrogen bond interactions but also hydrophobic contacts between ligand and main or subchain elements of receptor proteins as shown in figure below.

4.6.1 ER Ligand Complexes Analysis using LigPlot

Figure 4.4 shows interaction of α-terpinene with ER protein. It depicts that α-terpinene has formed 6 hydrophobic interactions.

Figure 4.5 shows the interaction of Asarinin with ER protein. It indicates that Asarinin made 9 hydrophobic interactions.

Figure 4.6 shows the interaction of Beta phallandrene with ER protein. It shows that Beta phallandrene made 8 hydrophobic interactions.

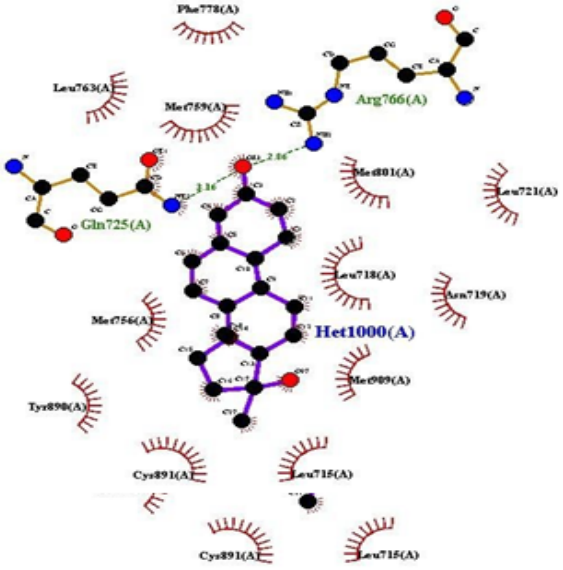


FIGURE 4.4: Interaction of αterpinene with receptor protein ER

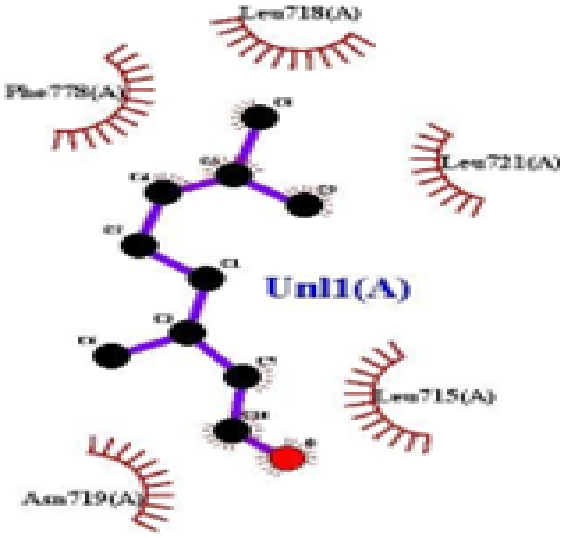


FIGURE 4.5: Interaction of Asarinin with receptor protein ER

Figure 4.7 shows the interactions of Borneol with ER. It indicates that Borneol formed 5 hydrophobic interactions. It also indicates the formation of 4 Hydrogen bonds.

Figure 4.8 shows the interactions between Camphene and ER. It also indicates the presence of 9 hydrophobic interactions of Camphene.

Figure 4.9 indicated the interactions between Isobutyl-vinylacetate and ER. It shows that this ligand forms 7 hydrophobic interactions and 2 Hydrogen bonds.

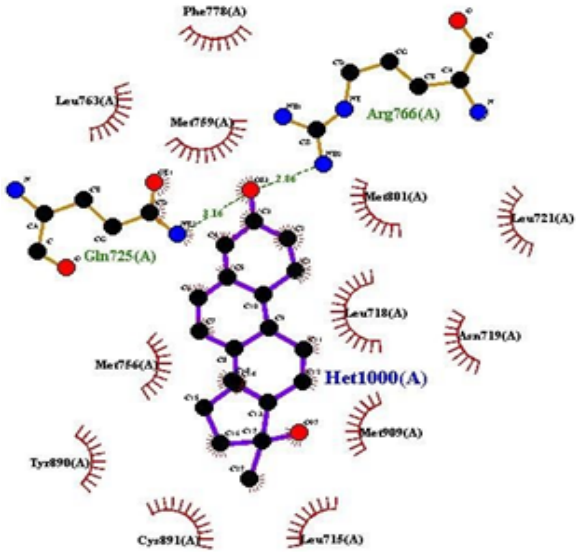


FIGURE 4.6: Interaction of βphallandrene with receptor protein ER

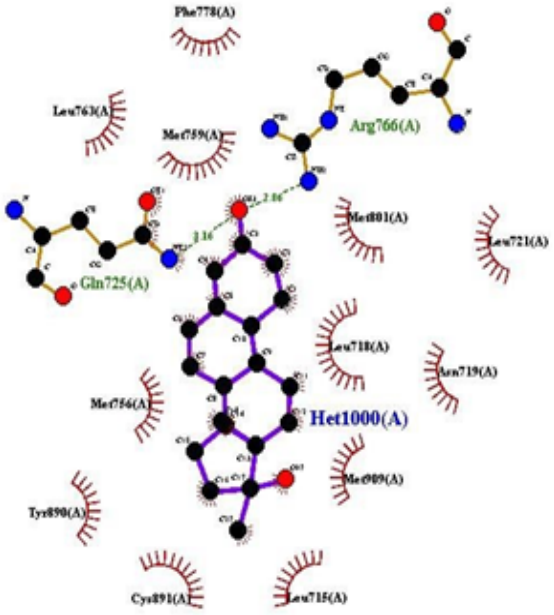


FIGURE 4.7: Interaction of Borneol with receptor protein ER

Figure 4.10 indicates the interaction of Myrcene with ER. It made 8 hydrophobic interactions.

Figure 4.11 shows the interaction between Neral and ER. Neral formed 10 hydrophobic interactions and 1 Hydrogen bond.

Figure 4.12 shows the interactions between p-cymene and ER. It formed 6 hydrophobic interactions.

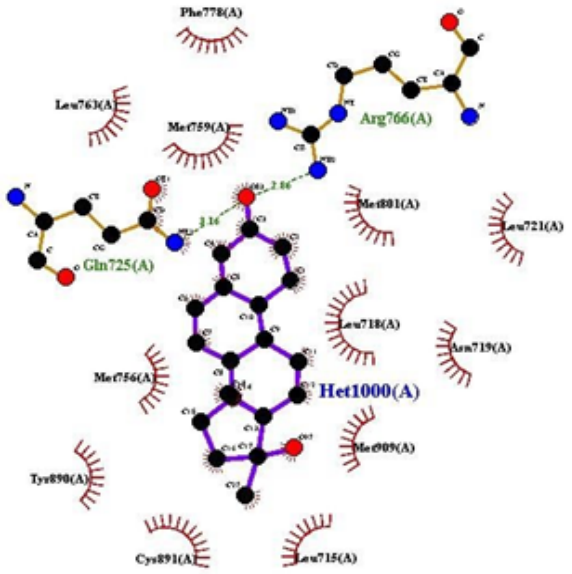


FIGURE 4.8: Interaction of Camphene with receptor protein ER

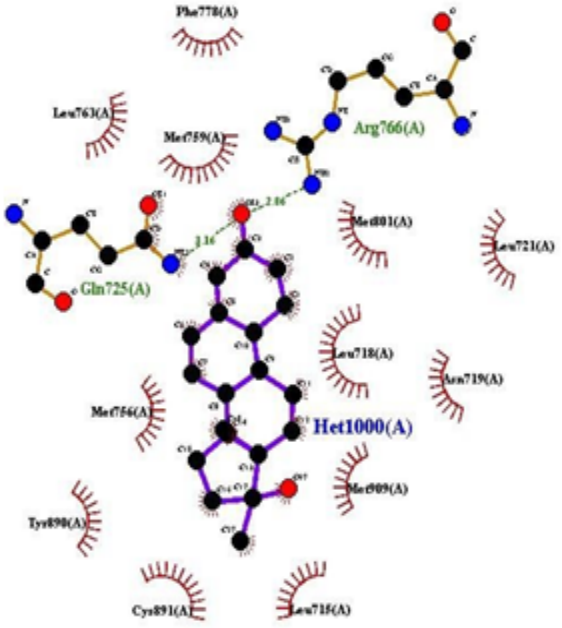


FIGURE 4.9: Interaction of Isobutylvinyl acetate with receptor protein ER

4.6.2 Ki-67 Complexes with Ligands

Figure 4.13 illustrates the detailed interactions between Quercitin and the Ki-67 protein. It reveals the formation of 2 hydrogen bonds along with 12 hydrophobic interactions, providing insights into the binding mechanism.

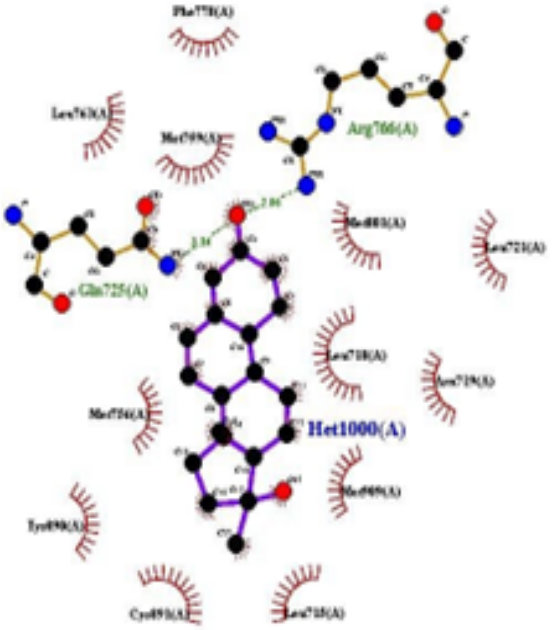


FIGURE 4.10: Interaction of Myrcene with receptor protein ER

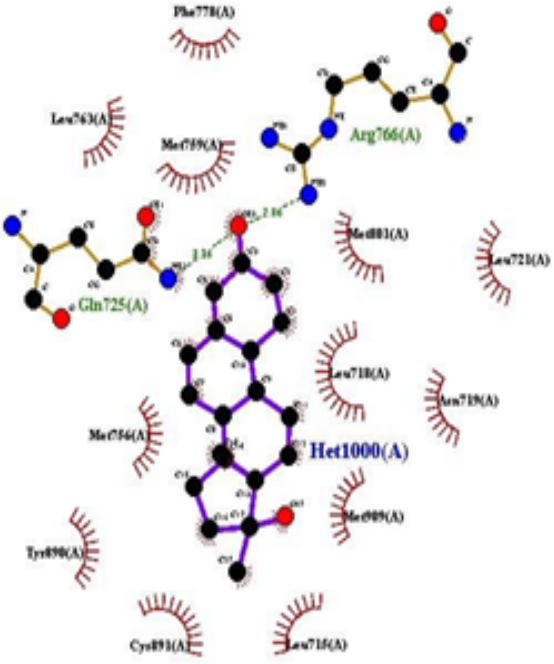


FIGURE 4.11: Interaction of Neral with Receptor protein ER

Figure 4.14 shows the interactions between Asarinin and Ki-67 protein. Asarinin formed 5 hydrophobic interactions.

Figure 4.15 shows the interactions between p-cymene and receptor protein Ki-67. Pcymene formed 12 hydrophobic interactions and 2 Hydrogen bonds.

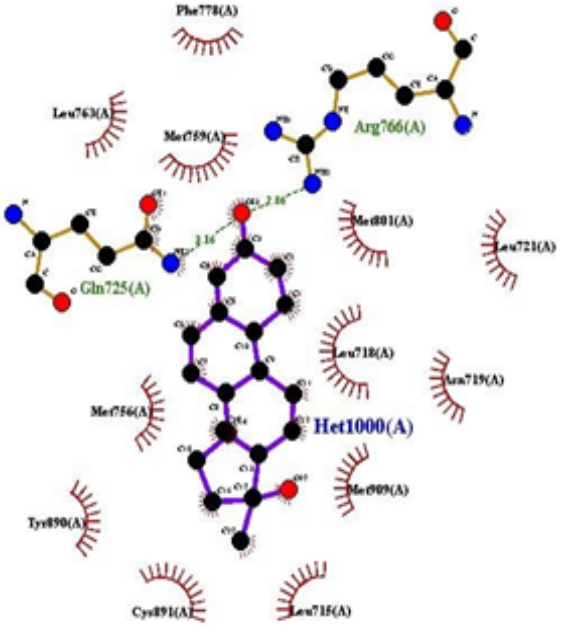


FIGURE 4.12: Interaction of p-cymene with Receptor protein ER

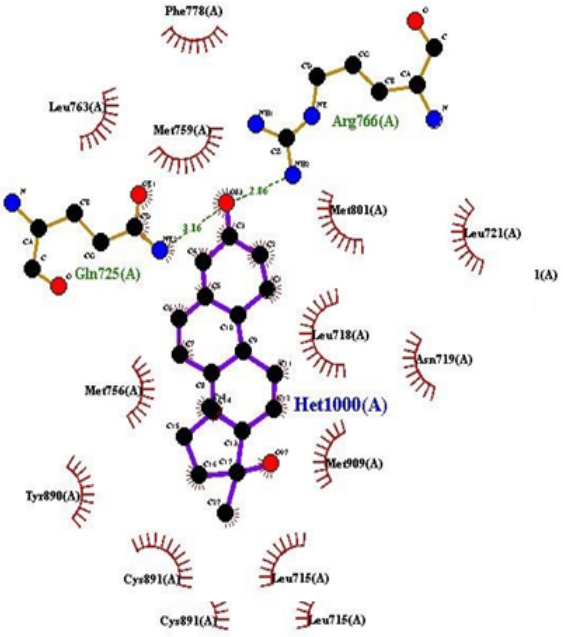


FIGURE 4.13: Interactions between Quercitin and Receptor protein Ki-67

Figure 4.16 illustrates the detailed interactions between Myrcene and the receptor protein Ki-67, providing valuable insights into their binding mechanism and potential therapeutic implications.

Figure 4.17 indicates the interactions between Neral and Ki-67 receptor protein. Neral formed 12 hydrophobic interactions and 2 Hydrogen bonds.

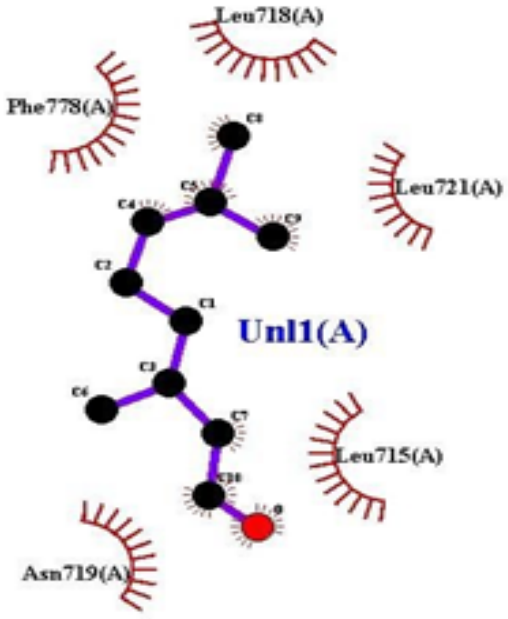


FIGURE 4.14: Interactions between Asarinin and Ki-67

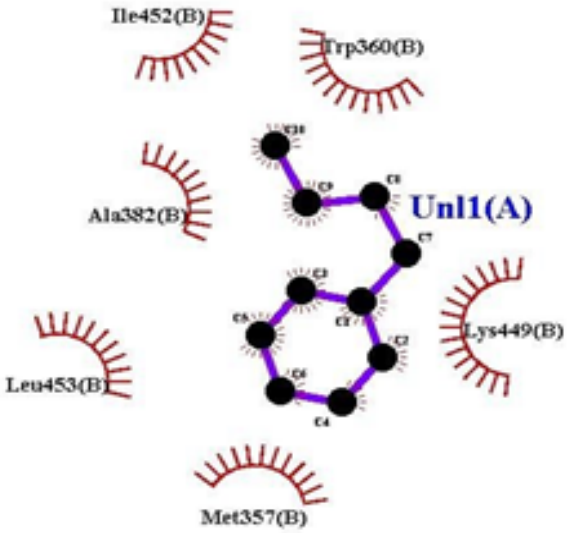


FIGURE 4.15: Interactions between p-cymene and receptor protein Ki-67

Figure 4.18 shows the interactions between Isobutylvinyl acetate and Ki-67. It formed 2 Hydrogen bonds and 12 hydrophobic interactions.

Figure 4.19 illustrates the intricate interactions between Borneol and the Ki-67 receptor protein. The analysis reveals the formation of 2 hydrogen bonds and 12 hydrophobic interactions, shedding light on the molecular mechanisms underlying their binding dynamics.

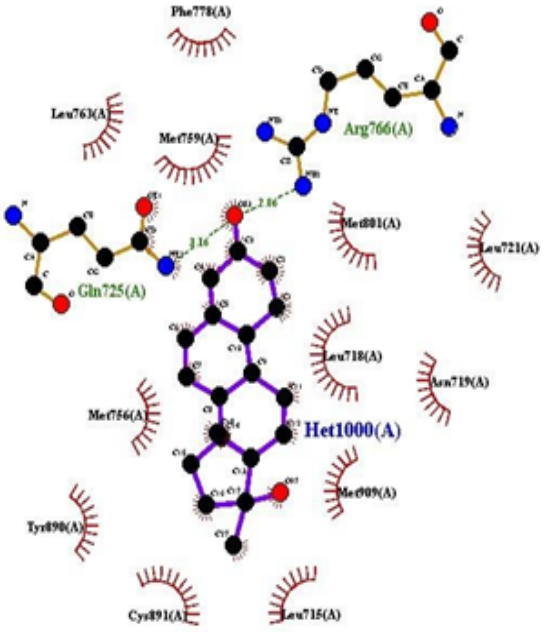


FIGURE 4.16: Interactions between Myrcene and Receptor protein Ki-67

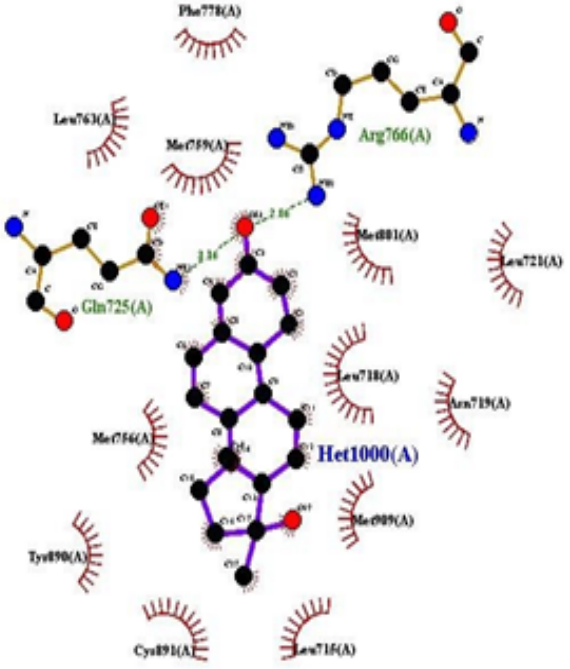


FIGURE 4.17: Interaction between Neral and Receptor protein Ki-6

Figure 4.20 shows the interactions between Camphene and Ki-67 protein. It formed 2 Hydrogen bonds and 12 hydrophobic interactions.

Figure 4.21 shows the interactions between β-phallandrene and Ki-67. It formed 12 Hydrophobic interactions. It formed 2 Hydrogen bonds.

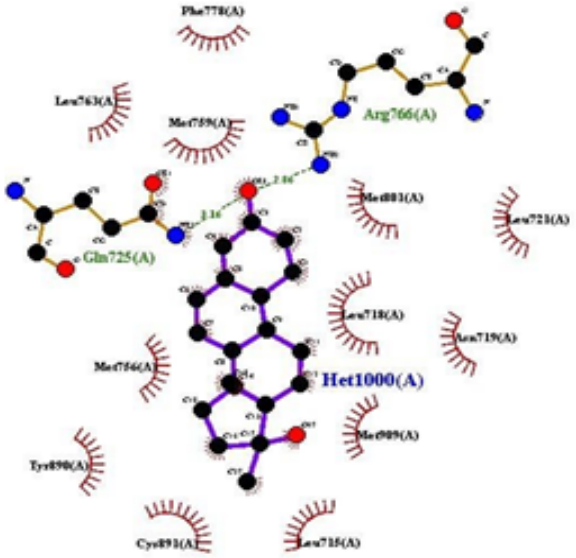


FIGURE 4.18: Interactions between Isobutylvinyl acetate and Receptor Protein Ki-67

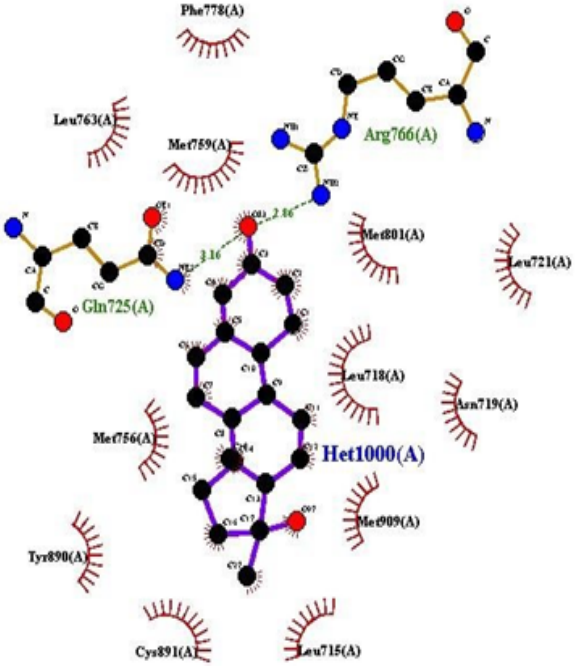


FIGURE 4.19: Interactions between Borneol and Receptopr protein Ki-67

4.7 ADME Properties of Selected Ligands

ADMET properties are very essential to study the nature of compounds. They provide profound information to predict the functionality and efficacy of a compound. Their mechanism of actions becomes more clear and defined with a comprehensive analysis of these properties.

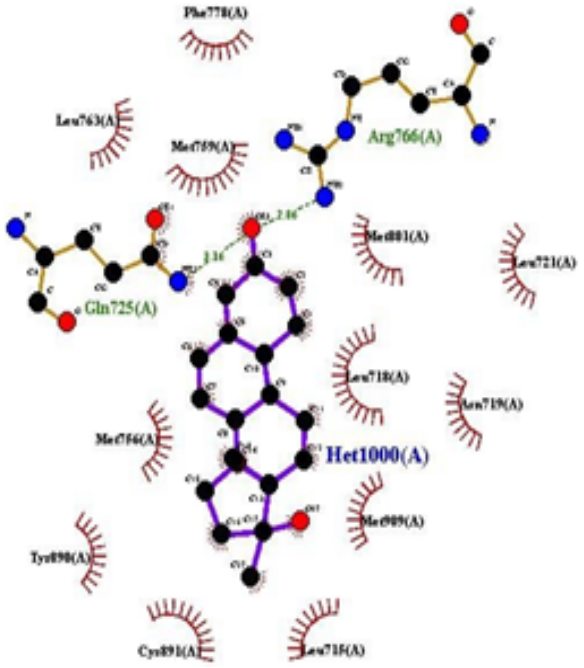


FIGURE 4.20: Interactions between Camphene and Ki-67 Receptor protein

4.7.1 Absorption

Table 4.12, shows that Camphene is most hydrophilic compound and holds highest solubility among other ligands under consideration. Whereas, Isopropyl vinyl acetate is least water soluble but is maximum absorbed in intestine. None except Quercitin acts as P Glycoprotein substrate. All other ligands neither act as P Glycoprotein I Inhibitors nor P Glycoprotein II inhibitors. Absorption properties of these compounds are reported by other scientists as well[44].

Table 4.13, shows that myrcene is most water soluble where as neral is least water soluble among given Ligands. Asarinin showed calcium carbonate maximum permeability and myrcene has minimum Calcium Carbonate permeability. Asarinin holds maximum intestinal absorption as compare to other Ligands under consideration. Skin permeability results made asarinin as better Candidate with highest scores. Following previous trend, myrcene has least Skin permeability. Asarinin acts as p Glycoprotein inhibitor rest all ligands neither act as p Glycoprotein I and II inhibitors nor substrate. Physical and absorption properties of Myrcene and Asarinin had been studied by other scientist as well[45].

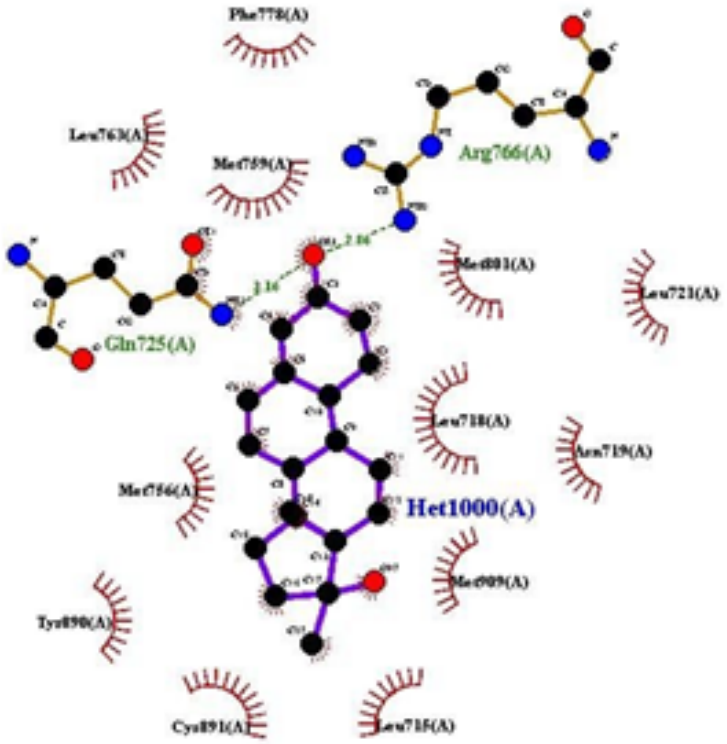


FIGURE 4.21: Interactions between Beta phallandrene and Receptor Protein Ki-67

4.7.2 Distribution

In Table 4.14, the comparison of distribution properties reveals insights into the behavior of the ligands in biological systems. The Virtual Deep Seismic Sounding values provide information on the volume of distribution, indicating the extent to which the ligands are distributed throughout the body.

Results from Table 4.14 showed that Volume of distribution in steady state or volume that is must to contain the total amount of drug in the body at the same concentration observed in plasma. It is an essential parameter reflecting the extent of a drug distribution throughout the body. P cymene shows moderate value of drug distribution which indicates that this drug candidate shall distribute moderately throughout the body and is not confined solely to the plasma. VDss values must be positive , negative value as in case of Isopropylvinylacetate indicates that this candidate shall not be distributed in the body as expected which could be

TABLE 4.12: Comparison of Absorption properties of Ligands 1-5

Absorption properties	**Borneol**	**pcymene**	**Camphene**	**Quercitin**	**Iso-vinyl propylacetate**
Water solubility	-2.826	-3.942	-4.34	-3.152	-1.378
CaCO2 permeability	1.501	1.518	1.387	1.021	1.387
Intestinal absorption	93.705	93.756	94.148	75.335	96.849
Skin permeability	-1.559	-1.089	-1.435	-2.735	-2.171
p-glycoprotein substrate	No	No	No	Yes	No
p-glycoprotein inhibitor	No	No	No	No	No
p- glycoprotein II inhibitor	No	No	No	No	No

TABLE 4.13: Comparison of Absorption properties of Ligands 6-10

Absorption properties	**βPhallan -drene**	**Neral**	**Myrcene**	**αterpinene**	**Asarinin**
Water solubility	-3.849	-2.99	-4.481	-4.012	-4.158
CaCO2 permeability	1.414	1.251	1.405	1.407	1.567
Intestinal absorption	96.548	96.16	95.393	95.778	98.955
Skin permeability	-1.508	-2.429	-1.058	-1.426	-2.785
p-glycoprotein substrate	No	No	No	No	No
p-glycoprotein inhibitor	No	No	No	No	Yes
p-glycoprotein II inhibitor	No	No	No	No	No

probably due to measurement error or unusual pharmacokinetic behavior. These VDss properties are also reported by other scientists[46].

Table 4.15 indicates the comparison of remaining Ligands under consideration. α-terpinene displayed moderate value of Distribution volume comparatively. Asarinin showed negative value that indicated some error or unusual behavior of Asarinin pharmacokinetics. β-Phallandrene shows moderate to high distribution of drug throughout the body.

TABLE 4.14: Comparison of Distribution Properties of the Ligands 1-5

Distribution traits	**Borneol**	**pcymene**	**Camphene**	**Quercitin**	**Isopropyl -vinyl- acetate**
VDss	0.172	0.67	0.547	0.223	-0.047
Fraction unbound (human)	0.443	0.202	0.354	0.061	0.585
BBB Permeability	0.615	0.554	0.787	-1.337	0.414
CNS Permeability	-2.179	-1.455	-1.71	-3.475	-2.499
p-glycoprotein substrate	No	No	No	No	No
p-glycoprotein inhibitor	No	No	No	No	Yes
p-glycoprotein II inhibitor	No	No	No	No	No

TABLE 4.15: Comparison of Distribution Properties of Ligands 6-10

Distribution traits	**β- phallandrene**	**Neral**	**Myrcene**	**α- terpinene**	**Asarinin**
VDss	0.408	0.118	0.368	0.421	-0.216
Fraction unbound	0.427	0.428	0.386	0.416	0.054
BBB Permeability	0.761	0.636	0.786	0.753	-0.378
CNS Permeability	-2.049	-0.2029	-1.912	-2.029	-2.79
p-glycoprotein substrate	No	No	No	No	No
p-glycoprotein inhibitor	No	No	No	No	Yes
p-glycoprotein II inhibitor	No	No	No	No	No

It implies that drug candidate is distributed beyond the plasma and into the tissues to a significant extent. Neral displayed 0.118 value which is relatively low distribution of drug throughout the body.

This could imply that the drug candidate shall largely confine to the plasma and do not penetrate tissues as extensively as drugs with higher VD values. These distribution properties are also studied by some other researchers[47].

TABLE 4.16: Comparison of Metabolic properties of Ligands 1-5

Metabolism traits	**Borneol**	**pcymene**	**Camphene**	**Quercitin**	**Isopropyl -vinyl- acetate**
CYP2D6 substrate	No	No	No	No	No
CYP3A4N substrate	No	No	No	No	No
CYP1AC inhibitor	No	Yes	No	Yes	No
CYP2C19 inhibitor	No	No	No	Yes	No
CYP2C9 inhibitor	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No
CYP34A inhibitor	No	No	No	No	No

4.7.3 Metabolism

Ligands that act as CYP2D6 or CYP3A4 substrates shall undergo metabolism by these enzyme. They are typically transformed into metabolites through oxidation which is a crucial step in drug clearance from the body. Substrates can exhibit varying degrees of metabolism by CYP2D6 and CYP3A4, ranging from extensive to poor metabolizers depending on genetic factors and drug interactions. Generally, drugs metabolized by CYP2D6 may have their pharmacokinetic properties altered in individuals with genetic polymorphism affecting CYP2D6 activity leading to variations in Drug efficacy and toxicity.

Ligands acting as inhibitors of CYP2D6 and CYP3A4 suppress the activities of these enzymes, leading to reduced metabolism of other drugs that are substrates of these enzymes. Inhibition of CYP2D6 and CYP3A4 can result in drug-drug interactions , where the presence of the inhibitors lead to increased concentrations of CYP2D6 and CYP3A4 substrates, potentially causing adverse effects. Quercitin acts as inhibitor of CYP1AC and CYP2C19 whereas p-cymene acts as CYP1AC inhibitor. Metabolism properties of these and other ligands are studied by different scientists[47]. Table 4.16 shows the metabolic properties of ligands.

TABLE 4.17: Comparison of Metabolic properties of Ligands 6-10

Metabolism traits	**β-phallandrene**	**Neral**	**Myrcene**	**α-terpinene**	**Asarinin**
CYP2D6 substrate	No	No	No	No	No
CYP3A4N substrate	No	No	No	No	Yes
CYP1AC inhibitor	No	No	No	No	Yes
CYP2C19 inhibitor	No	No	No	No	Yes
CYP2C9 inhibitor	No	No	No	No	Yes
CYP2D6 inhibitor	No	No	No	No	Yes
CYP34A inhibitor	No	No	No	No	Yes

Table 4.17, indicates that β-phallandrene, neral, myrcene and α-terpinene neither act as CYP2D6,CYP3A4,CYP1AC,CYP2C19,CYP2CP inhibitor nor substrate. However, asarinin acts as CYP3A4 substrate and inhibitor against CYP1AC, CYP2C19, CYP2C9, CYP2D6 and CYP34A. Asarinin shall undergo metabolism by CYP2D6 enzyme. They are typically transformed into their metabolites via oxidation reaction. Despite this property Asarinin exhibits significant inhibition to other enzymatic receptors under consideration.

Asarinin, with its broad-spectrum inhibitory effects on drug metabolism, presents significant implications for clinical practice, drug development, and patient care.

These inhibitors interact with active or allosteric sites of metabolic enzymes, effectively reducing their activity. Being multiple cytochrome inhibitors, these compounds disrupt various metabolic pathways involved in drug and endogenous substance biotransformation. This interference can lead to altered pharmacokinetics, potentially causing drug interactions, increased drug concentrations, and altered drug efficacy or toxicity [47].

Understanding the ADME properties of such compounds is crucial for predicting their behavior in vivo, guiding drug development, and optimizing therapeutic regimens.

TABLE 4.18: Comparison of Excretion properties of Ligands 1-5

Excretion traits	**Borneol**	**pcymene**	**Camphene**	**Quercitin**	**Isovinyl propylacetate**
Total Clearance	0.437	0.294	0.049	0.663	0.964
Renal OCT2 substrate	No	No	No	No	No

4.7.4 Excretion

Comparing excretion pathways in terms of total clearance values and renal OCT2 substrate involvement provides insights into drug elimination and potential interactions with the organic cation transporter 2 system. Total clearance represents the overall rate at which drug is removed from the body, encompassing all elimination pathways, including renal 0065 cretion, hepatic metabolism, and other clearance mechanisms. It is expressed in units of volume per unit time. High total clearance value indicate rapid elimination of the drug from the body, while low values suggest slower elimination. Organic cation transporter 2 is an important transporter located in the renal tubules responsible for the uptake of organic cations including many drugs from the blood into the kidney for excretion[48].

Drugs that are substrates for OCT2 are actively transported from the blood into the renal tubular cells, facilitating their elimination into the urine. Borneol indicates 0.437 value which is the rate at which the this compound is removed from the body. It represents the sum of all processes involved in the elimination of the compound, including metabolism and excretion. The total clearance value of p-cymene is comparatively low as compared to Borneol. This indicates that p-cymene shall be eliminated from the body at a slower rate. Camphene shows total clearance value of 0.049 that portrays relatively low rate of elimination. It would be removed from the body at a slower pace compared to previous compounds. Quercitin shows 0.663 value which is moderate, suggesting that it would be eliminated from the body at a moderate rate. Isovinylpropylacetate holds value 0.964

TABLE 4.19: Comparison of Excretion properties of Ligands 6-10

Excretion traits	**β-phallandrene**	**Neral**	**Myrcene**	**α-terpinene**	**Asarinin**
Total Clearance	0.196	0.376	0.438	0.223	-0.104
Renal OCT2 substrate	No	No	No	No	No

which highest among others. It indicates that such compounds shall rapidly eliminated from the body. Table 4.18 indicates the comparison of excretion properties of Ligands.

Table 4.19 shows that myrcene holds highest TC value which makes its elimination rapid from the body. Neral and α-terpinene depicted moderate rate of elimination from the body. However, β-phallandrene exhibited slower rate of elimination from the body. Total clearance values are supposed to have a positive value[48]. Negative value indicates the unusual behavior of Asarinin. None of the above mentioned ligands acted as the Renal OCT2 substrate.

4.8 Hydrogen and Hydrophobic Interactions in PR-Ligand Complexes

Hydrogen and hydrophobic interactions are very important to develop the relationship between a receptor and substrate protein. Counting and analyzing hydrogen bonds and hydrophobic interactions in protein-ligand complexes are essential for understanding the molecular mechanisms of Ligand binding and recognition. It helps in rational drug design, where modifying ligand structures to optimize hydrogen bonding and hydrophobic interactions can enhance the binding affinity and selectivity for the target protein. In protein-ligand complexes hydrogen bonds are formed between specific amino acid residues in the protein and functional groups on the ligand. Hydrophobic interactions arise from tendency of non-polar molecules or groups to minimize contact with water molecules. These hydrophobic interactions are formed between non polar regions of the ligand and hydrophobic

TABLE 4.20: Active Ligands showing Hydrogen and hydrophobic interactions with PR

Ligand name	**No. of H bonds**	**Amino acids in HB**	**Distance**	**Hydrophobic bonding**
Borneol	2	Arg766A Gln725A	2.14 3.16	12
P-cymene	2	Arg766A Gln725A	2.14 3.16	12
Camphene	2	Arg766A Gln725A	2.14 3.16	12
Quercitin	2	Arg766A Gln725A	2.14 3.16	12
Isobutyl-vinylacetate	0	-	-	6
β-phallandrene	2	Arg766A Gln725A	2.14 3.16	12
Neral	0	-	-	5
Myrcene	2	Arg766A Gln725A	2.14 3.16	12
α-terpinene	2	Arg766A Gln725A	2.14 3.16	12
Asarinin	2	Lys822B Trp765B	3.84 3.17	9

amino acid residues in the protein. These interactions help to stabilize the binding of hydrophobic ligands to protein pockets. These interactions contribute to the overall binding affinity and specificity of the protein-ligand complex.

Physicochemical properties of above mentioned compounds in table 4.20 had already studied by some scientists such as quercitin and β-phallandrene role in therapeutic agents derived from plants[49].

4.9 Comparison with Standard Drug Tamoxifen

To predict the future choice of drug formulation, the properties of active ligands were compared with those of Tamoxifen, a standard drug used in breast cancer treatment. Their physicochemical properties, including solubility, stability, and molecular weight, were contrasted with those of Tamoxifen. Additionally, their

docking behavior with receptor proteins was analyzed to assess their potential efficacy and specificity compared to Tamoxifen.

This comparison serves as a crucial step in evaluating the potential of these ligands as alternative or complementary treatments to Tamoxifen. By understanding how these ligands compare to a known standard, researchers can make informed decisions regarding their suitability for further development and clinical use in breast cancer therapy.

4.9.0.1 Molecular Docking of Tamoxifen with Target Proteins

Tamoxifen has two hydroxyl groups and a ring tail that lacks oxygen and contains nitrogen. Tamoxifen causes mutations by binding to the binding domain of the estrogen receptor. This mutation transferred helix 12 to the adjacent coactivator. This area is very important for the function of estrogen. Without connecting the coactivator, the receiver remains inactive. Changes also occur due to new hydrophobic interactions between helices 3 and 11. These new hydrophobic interactions disrupt the changes throughout the system. When one of the tamoxifen rings is pushed into the pocket, the new side chain also changes. Tamoxifen also forms smaller hydrogen bonds within the pocket than estrogen, causing elongation of helices 3, 8, and 11. When the coactivator sites are blocked, growth stops and therefore cell proliferation does not occur[50].

4.9.1 Physicochemical Properties of Tamoxifen

- Molecular weight: 371.51 g/mol
- H-bond acceptors: 2
- H-bond donor: 0
- Number of rotatable bonds: 8
- Log P: 5.9961

4.9.2 ADMET Properties of Tamoxifen

The properties of ADMET have already been studied by many scientific researchers who declared ADMET as the gold standard drug against early stage breast cancer. Mishaps were then reported and better alternatives were combined. Despite major advances in the understanding and management of breast cancer over the past decades, the disease remains a serious public health challenge and poses a significant burden worldwide. Selective estrogen modulators (SERMs) such as tamoxifen are approved for the treatment of this disease, but the development of drug resistance and adverse side effects such as endometrial cancer due to long-term chemotherapy with tamoxifen have limited treatment. Therefore, there is a strong need to develop new ER+ drugs with better therapeutic efficacy[25].

4.9.2.1 Absorption

- Water Solubility: $10^{-6.118}$ log mol/L
- $CaCO_2$ permeability: 1.088
- Human intestinal absorption: 35.562
- Skin permeability: $10^{-2.729}$
- P-glycoprotein substrate: Yes
- P-glycoprotein I inhibitor: Yes
- P-glycoprotein II inhibitor: Yes

4.9.2.2 Distribution

- VDss human: 0.883 log L/kg
- Fraction Ultrasound: 0.091 FU
- BBB Permeability: $10^{1.342}$ log BB
- CNS Permeability: $10^{-1.325}$ log PS

4.9.2.3 Metabolism

- CYP2D6 Substrate: Yes
- CYP3A4 Substrate: Yes
- CYP1A2 Inhibitor: Yes
- CYP2C19 Inhibitor: No
- CYP2C9 Inhibitor: No
- CYP2B6 Inhibitor: Yes
- CYP3A4 Inhibitor: Yes

4.9.2.4 Excretion

- Total Clearance: 0.616 log mL/min/kg
- Renal OCT2 Substrate: No

4.9.3 Toxicity

- AMES Toxicity: Yes
- Max. tolerated dose adult human: 0.341
- NERG I inhibitor: Yes
- NERG II inhibitor: Yes
- Oral Rac Acute Toxicity: 2.258 Oral
- Oral Rac Chronic Toxicity: 0.358
- Hepatotoxicity: No
- Skin sensitization: No

4.10 Tamoxifen Mechanism of Action

Tamoxifen competitively prevents the binding of estrogen to receptors. This is important for estrogen activity in breast cancer cells. Tamoxifen decreases tumor growth factor alpha and insulin-like growth factor 1 and increases sex hormone-binding globulin. An increase in sex hormone-binding globulin limits the amount of free estradiol. These changes reduce the levels of factors that stimulate tumor growth[51].

Tamoxifen has also been shown to induce apoptosis in estrogen receptor-positive cells. This effect is thought to be the result of inhibition of protein kinase C, which prevents DNA synthesis. Another theory for the apoptotic effects of tamoxifen is due to an approximately 3-fold increase in intracellular and mitochondrial calcium ion levels after administration or induction of tumor growth factor β [30].

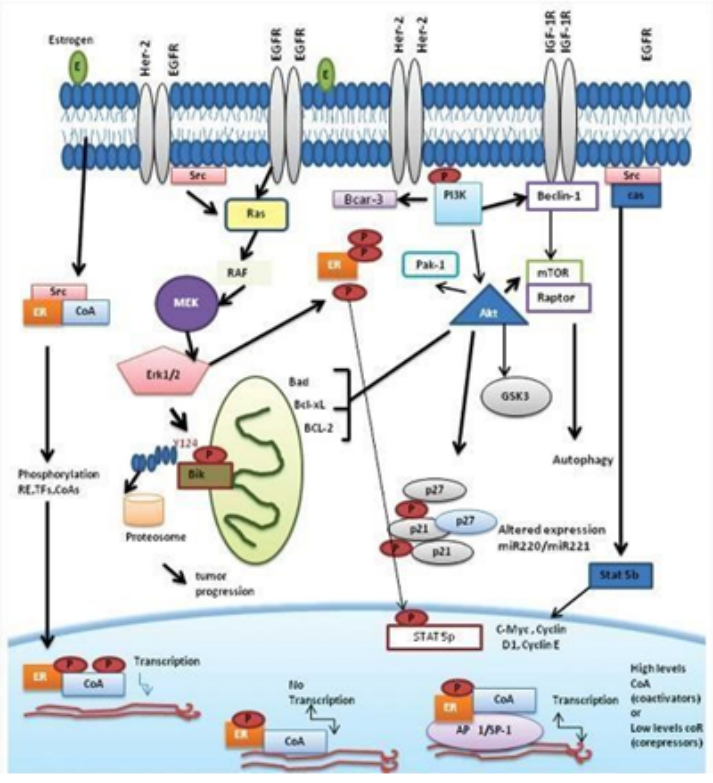


FIGURE 4.22: Molecular mechanism of action of Tamoxifen as an ER inhibitor[33]

Tamoxifen exerts estrogen agonist and antagonist effects in various parts of the body as shown in figure 4.22. It selectively binds to estrogen receptors, causing

both estrogenic and antiestrogenic effects; Since it has two effects, it is a patient-specific selective estrogen receptor modulator (SERM). Breast cancer is one of the most common cancers among women worldwide. Tamoxifen has been used as endocrine therapy for breast cancer for the past 40 years. This leads to a 30% reduction in death rates and is also one of the most effective treatments against cancer. However, resistance to tamoxifen is still an important problem in breast cancer treatment. A lot of research has been done in the past decade to explore its protective mechanism, but more research is still needed to solve this problem. Various biochemical and molecular pathways, such as altering ER signaling and increasing growth factors, have been shown to be important in tamoxifen resistance (TR). After five to ten years of initial treatment, breast cancer patients can recover with these drugs. This resistance can lead to the development of other cancers, such as uterine cancer[51].

4.11 Tamoxifen Effects on Body

Tamoxifen is a selective estrogen receptor modulator that inhibits the growth and induces apoptosis of estrogen receptor-positive tumors. The active metabolite N-desmethyl tamoxifen has a half-life of approximately 2 weeks, resulting in a longer duration of action. The therapeutic index is narrow because high doses can cause respiratory problems and seizures. Tamoxifen administration is also associated with increased incidence of uterine malignancies[52].

4.12 Tamoxifen Action Pathway

Tamoxifen is a selective estrogen receptor modulator (SERM) used to treat estrogen-sensitive breast cancer. Tamoxifen itself has only weak antiestrogenic effects and must be converted to more active metabolites for therapeutic activity. Metabolism occurs in the liver and is mainly carried out by cytochrome P450 enzymes. Tamoxifen is hydroxylated by CYP2D6 and demethylated by CYP3A4 and CYP3A5

to produce the active metabolites 4-hydroxy tamoxifen and endoxifen. These metabolites inhibit estrogen binding to estrogen receptors in breast cancer cells, thereby inhibiting tumor growth [25][52].

4.13 Tamoxifen Docking Results

Tamoxifen was docked against 3 selected proteins indivisually to obtain binding affinity results. Tamoxifen showed maximum binding affinity with ER and PR proteins by getting vina score of -8.6. However, with Ki-67 binding affinity was found to be -7.3. SWISS ADME was used to study the ADME properties of Tamoxifen. It contained 26 Carbon atoms, 29 Hydrogen atoms, 1 Nitrogen and 1 Oxygen atom.

Figure 4.23 shows the interactions between Tamoxifen and PR. Tamoxifen indicates 12 hydrophobic interactions.

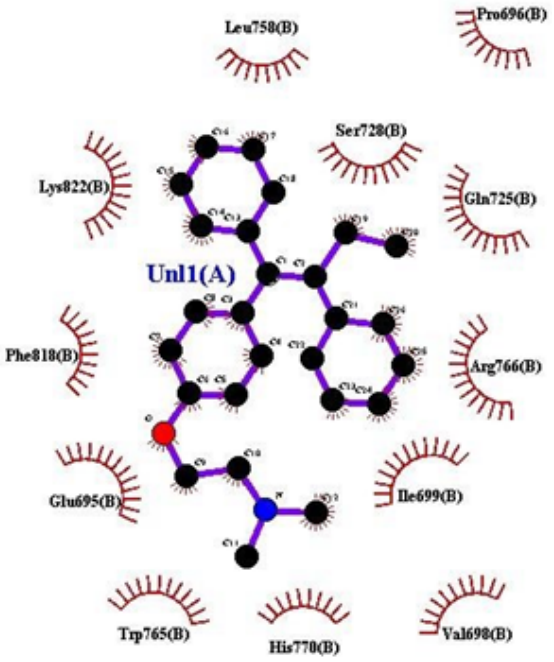


FIGURE 4.23: Interaction of Tamoxifen with PR receptor protein

Tamoxifen possesses a bulky hydrophobic core and an aromatic ring system. The hydrophobic nature of tamoxifen enables it to interact favorably with non-polar

residues in PR binding site. The binding site of progesterone receptor contains hydrophobic pockets and regions that can accommodate hydrophobic ligands. These pockets provide favorable environments for hydrophobic interactions with ligands like tamoxifen. In the absence of hydrogen bonds, hydrophobic interactions become predominant in stabilizing the tamoxifen-PR complex. In this complex the absence of hydrogen bonds may be due to the lack of suitable hydrogen bond donor or acceptor groups in tamoxifen or PR binding site. Despite the absence of hydrogen bonds , the hydrophobic interactions between tamoxifen and PR are sufficient to stabilize the complex[53].

Figure 4.24 indicates the interactions between Tamoxifen and Estrogen Receptor. It indicates the presence of 16 hydrophobic interactions.

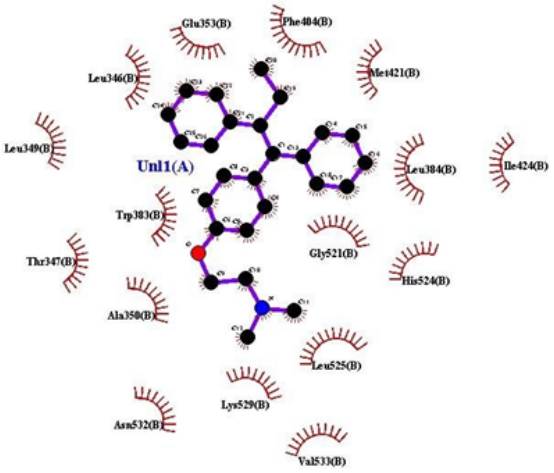


FIGURE 4.24: Interactions between Tamoxifen and Estrogen Receptor

As compare to PR complex with tamoxifen, ER-Tamoxifen complex contains 16 hydrophobic interactions making it more stable. Despite the absence of hydrogen bonds, the hydrophobic interactions between tamoxifen and ER are sufficient to stabilize the complex.

Figure 4.25 shows the interactions between the gold standard Tamoxifen and Ki-67 receptor protein.

The analysis revealed 13 hydrophobic interactions and 1 hydrogen bond between the ligand and the receptor protein. These interactions play a crucial role in determining the binding affinity and specificity of the ligand to its target protein.

Understanding these interactions is essential for elucidating the ligand's mechanism of action and predicting its therapeutic potential. Further investigation into specific residues involved can provide valuable insights for ligand optimization.

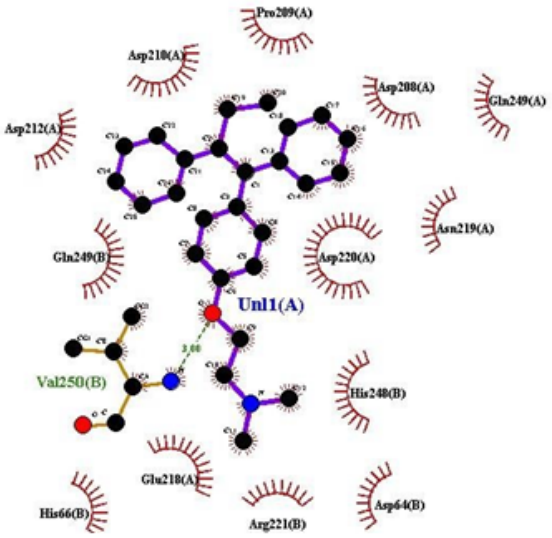


FIGURE 4.25: Interactions between Tamoxifen and Ki-67 receptor protein

Tamoxifen Ki-67 complex showed the presence of 1 hydrogen bond and 13 hydrophobic interactions. This interaction between Tamoxifen and Ki-67 protein is indirect. Tamoxifen inhibits estrogen signaling in estrogen receptor positive breast cancer cells, leading to the suppression of cell proliferation. As a result treatment with tamoxifen is predicted to reduce the expression of Ki-67 in breast cancer tumors, reflecting decreased cell proliferation rate. It is considered a favorable response and is associated with improved patient outcomes and decreased risk of disease recurrence[38].

4.14 Tamoxifen Comparison with the Lead Compounds

The comparison between Tamoxifen and the lead compounds involved assessing their physicochemical properties and docking behavior with the receptor protein. This analysis aids in identifying potential drug candidates.

4.15 ADMET Properties Comparison

The ADMET characteristics were compared to evaluate the absorption, distribution, metabolic excretion, and toxicity of the medication and lead molecule to identify a promising candidate.

4.16 Comparison Result of Absorption Properties

The six models utilized for determining absorption parameters play a critical role in drug assessment. The water solubility model, assessing solubility at 25°C, sets the baseline. Following oral intake, the CaCO2 solubility model gauges absorption efficacy, with readings surpassing 0.90 indicating heightened intestinal absorption. Asarinin demonstrates the highest absorption, surpassing quercetin and tamoxifen, although quercetin exhibits better absorption than tamoxifen. Values below 30% in the intestinal absorption model signify inadequate absorption.

Skin penetration, essential for transdermal drugs, considers values below log Kp >-2.5 as low, with all three compounds passing the test. The P-glycoprotein substrate model assumes significance due to P-glycoprotein's role as an ABC transporter, forming a biological barrier. Lastly, the P-glycoprotein inhibitors model discerns inhibitory properties. Tamoxifen and asarinin act as P-glycoprotein substrates, alongside quercetin.

Table 4.21: Trend of Absorption

Absorption Models	**Tamoxifen**	**Quercitin**	**Asarinin**
Water solubility	-6.118	-3.152	-4.158
$CaCO_2$ permeability	1.088	1.021	1.567
Intestinal absorption	35.562	75.335	98.955
Skin permeability	-2.729	-2.735	2.785
p-glycoprotein substrate	Yes	Yes	No
p-glycoprotein inhibitor	Yes	No	Yes
p-glycoprotein II inhibitor	Yes	No	No

Table ?? indicates the trend of absorption in tamoxifen, quercitin and asarinin.

4.17 Comparison Results of Distribution Properties

The distribution parameters are based on four models. The drug's uniform dispersion in the plasma is measured by the volume of distribution (VDss), and if this value is greater than 2.81 L/kg, the drug is more distributed in the tissues than in the plasma. All three compounds have reasonable VDss values. The second model is based on the proportion of unbound drug in plasma, as bound drug affects drug efficacy. The specified amount is the amount of medicine that remains indefinitely. For BBB permeability, if its value is greater than 0.3 logBB, the drug can easily cross the blood-brain barrier. If the value is less than -1 logBB, the drug is not distributed in the brain or is poorly distributed. According to table 4.20, the amount of tamoxifen is 1.342. However, the distribution of asarin and quercetin in the brain is bad.

The CNS model is based on a logPS value of > -2, indicating that the medication may easily reach the CNS. Drugs with a logPS value of < -3 are unable to infiltrate the CNS. Tamoxifen has slight chance to penetrate through the brain where as values for Quercitin and Asarinin indicate they shall not be able to penetrate CNS barriers. Table 4.22 shows that Tamoxifen has high value of Distribution volume as compared to lead compounds. A high VDss suggests extensive distribution beyond the blood stream. Tamoxifen typically has a high VDss due to its extensive tissue distribution, particularly in breast tissue. Quercitin and asarinin have varying VDss values due to the difference in their affinity for tissue and plasma proteins. Drugs with low protein binding tend to have high fractions unbound. A higher fraction unbound increases the likelihood of distribution into tissues and across barriers. Quercitin and Asarinin being polyphenolic , may have moderate to high fractions unbound, facilitating their distribution. Tamoxifen has limited BBB permeability, which restricts its access to CNS. Quercitin and asarinin being

TABLE 4.22: Comparison of Distribution models of Tamoxifen and Lead compounds

Distribution models	**Tamoxifen**	**Quercitin**	**Asarinin**
VDss	0.883	0.223	-0.216
Fraction unbound (human)	0.091	0.061	0.054
BBB Permeability	1.342	-1.3711	-0.378
CNS Permeability	-1.3251	-3.475	-2.79

smaller molecules and potentially more lipophilic have better BBB permeability, allowing them to enter CNS[54].

in table 4.22, Tamoxifen tends to have a high VDss with limited CNS penetration due to poor BBB permeability. Quercitin and asarinin may have moderate to high VDss with potential CNS penetration , facilitated by their higher fractions unbound and potentially better BBB permeability compared to tamoxifen. These factors collectively influence the distribution properties of these compounds within the body[38][54].

4.18 Comparison Results of Metabolism Properties

Cytochrome P450 is found mainly in the liver and is responsible for oxidizing xenobiotics so that they can be easily eliminated from the body, turning cytochrome P450 into a detoxifying enzyme. Some drugs are activated and some are deactivated as a result. Therefore, it is important to assess whether a compound is a P450 substrate or a P450 inhibitor[55]. Table 4.23 indicates the comparison of metabolic properties of our standard drug Tamoxifen and two lead compounds. It shows that Tamoxifen acts as a substrate as well as inhibitor to all receptor compounds under consideration.

Table 4.23 illustrates the metabolic interactions of Tamoxifen, Quercetin, and Asarinin with various cytochrome P450 enzymes. Tamoxifen demonstrates a complex profile, serving as both a substrate for CYP2D9 and CYP3A4N, and as an

TABLE 4.23: Comparison of Metabolism models of Tamoxifen and Lead compounds

Metabolism models	**Tamoxifen**	**Quercitin**	**Asarinin**
CYP2D6 substrate	Yes	No	No
CYP3A4 substrate	Yes	No	Yes
CYP1A C inhibitor	Yes	Yes	Yes
CYP2C19 inhibitor	No	Yes	Yes
CYP2C9 inhibitor	No	No	Yes
CYP2D6 inhibitor	Yes	No	Yes
CYP34A inhibitor	Yes	No	Yes

inhibitor for CYP1AC, CYP2D6, and CYP34A. Quercetin, on the other hand, acts as an inhibitor for CYP1A and CYP2C19. Asarinin exhibits a similar inhibitory pattern to Quercetin, acting as a substrate for CYP3A4N, and as an inhibitor for CYP1AC, CYP2C19, CYP2C9, CYP2D6, and CYP34A. These interactions highlight the diverse roles these compounds play in modulating metabolic pathways, underscoring their potential therapeutic significance in drug development and patient care.

4.19 Comparison Results of Excretion Properties

Excretion models describe how drug or drug candidate shall be eliminated from the body via various processes such as renal excretion, hepatic metabolism, and fecal elimination. Excretion pattern of drugs under consideration indicates that Tamoxifen has relatively low total clearance which suggests its moderate elimination from the body. It likely undergoes hepatic metabolism and renal excretion with a considerable portion eliminated through bile and feces. The value of total clearance is a combination of hepatic and renal clearance and is important so that the dose rates of the drugs can be assessed. The 2nd model is the Renal OCT2 (organic cation transporter 2) and this transporter helps in the renal clearance of drugs and other compounds. Being an OCT2 substrate can have an adverse effect in correlation with inhibitors[56].

TABLE 4.24: Comparison of Excretion models of Tamoxifen and Lead compounds

Excretion models	**Tamoxifen**	**Quercitin**	**Asarinin**
Total Clearance	0.616	0.663	-1.04
Renal OCT2 substrate	No	No	No

Quercitin with a slightly higher total clearance than tamoxifen may undergo similar elimination but at a slightly faster rate. It may also undergo significant renal excretion owing to its relatively low molecular weight and water solubility. The negative total clearance value for asarinin seems anomalous and indicate an error. If such a value is attained after replicating entire process it shall advocate that drug concentration in the body increases over time instead of decreasing[57].

Table 4.24 indicates that Quercitin has more total clearance as compare to Tamoxifen and Asarinin. All three compounds are not Renal OCT2 substrates.

4.20 Comparison Results of Toxicity Properties

Standard drug toxicity and lead composition were based on nine models. AMES toxicity model 1 shows that both standard and lead compounds are not mutagenic. In model 2 for the maximum tolerated dose, values below 0.477 log mg/kg/day are considered low and higher values are considered high. The table below shows the low tolerable doses of tamoxifen. The third model is hERG I and II inhibitors and both of these compounds are not inhibitory. It is useful to assess the relative toxicity using the fourth rat acute oral toxicity paradigm.[57][58].

Model 5 of oral rat chronic toxicity calculates the lowest dose that could cause an adverse effect; Model 6 of hepatotoxicity analyzes whether the drug can influence the liver; and Model 7 of dermal products evaluates skin sensitivity. The standard and lead compounds do not cause skin sensitivity. Models 8 and 9 assess toxicity using T. Pyriformis and minnows, respectively; values > -0.5 for T. Pyriformis indicate toxicity, indicating that tamoxifen is somewhat toxic, and values below 0.5mM for minnows indicate toxicity; all three compounds pass this toxicity test.

TABLE 4.25: Comparison of Toxicity Models of Tamoxifen and Lead compounds

Traits	**Tamoxifen**	**Quercitin**	**Asarinin**
AMES Toxicity	Yes	No	Yes
Maximum tolerated dose	0.341	1.159	0.346
HERG-I inhibitor	Yes	No	No
HERG-II inhibitor	Yes	No	No
Oral Rat Acute Toxicity	2.258	1.944	2.876
Oral Rat Chronic Toxicity	0.358	3.169	1.619
Hepatotoxicity	No	No	No
Skin sensation	No	No	No
T-pyriformis toxicity	0.328	0.294	0.321
Minnow toxicity	0.095	1.305	-0.75

Tamoxifen's toxicity is well-documented across a number of parameters, including AMES toxicity, HERG inhibition, and hepatotoxicity. Tamoxifen has been reported to inhibit hERG channels which can lead to cardiac arrhythmias and QT prolongation, posing a risk of sudden cardiac death [58]. These findings highlight the importance of comprehensive toxicity assessments in drug development and safety evaluation.

Table 4.25 indicates the comparison of toxicity traits between Tamoxifen and Lead compounds. Results of model 1 indicate that Tamoxifen and Asarinin are both are mutagenic. Maximum tolerated dose values vary from Tamoxifen and Asarinin sharing low values where as slight high value is obtained for Quercitin. Tamoxifen is hERG I and II inhibitor where as both lead compounds are not hERG inhibitors. All three compounds are prone to liver toxicity. No skin sensation is reported in case of all three compounds.

4.21 Results of Physicochemical Properties Comparison

For determining the fundamental properties of these compounds their physicochemical properties were studied. SWISS ADME tool was used for this purpose. Through this screening from Table 4.26, we get to know that Tamoxifen contains 26

TABLE 4.26: Comparison of Physicochemical properties of Tamoxifen and Lead compound

Drug	**Molecular formula**	**H bond donor**	**H bond acceptor**	**Log P Value**	**Molecular Weight (g/mol)**	**Rotateable bonds**
Tamoxifen	Molecular formula	0	2	5.996	371.5	8
Asarinin	Molecular formula	0	6	3.2192	354.358	2

Carbon atoms, 29 Hydrogens, 1 Nitrogen and 1 Oxygen atom. Quercitin contains 15 Carbons, 10 Hydrogens and 7 Oxygen atoms. Asarinin contains 12 Carbons, 16 Hydrogens and 6 oxygens. This shows that our lead compounds are more simple as compared to Tamoxifen. Tamoxifen can donate no hydrogen atoms However, it can accept 2 Hydrogen atoms. There are 8 rotateable bonds present in Tamoxifen. Log P value of Tamoxifen is 5.996. Asarinin has log P value= 3.2192 . Asarinin can accept 6 Hydrogens and couldn't donate any.

Tamoxifen, a non-steroidal anti-estrogenic medication, serves as a cornerstone in breast cancer treatment. Its intricate chemical structure boasts multiple aromatic rings and functional groups, contributing to its pharmacological activity. In contrast, asarinin, a naturally occurring lignin abundant in various plant species such as *Asarum canadense*, typically comprises two phenylpropane units linked by a bond. With a molecular weight of 371.5 g/mol, tamoxifen outweighs asarinin, whose molecular weight may vary but generally leans lighter due to its simpler, naturally derived structure.

While tamoxifen exhibits limited solubility in water, it dissolves readily in organic solvents like ethanol and methanol, facilitating its formulation into various dosage forms. In comparison, asarinin's solubility, contingent on its chemical configuration and solvent, usually demonstrates moderate solubility in organic solvents and restricted solubility in water, posing challenges in formulation development.

TABLE 4.27: Comparison of Docking score of Tamoxifen and Lead compound

Compound	**Binding Score**	**H bond donor**	**H bond acceptor**	**Log P Value**	**Molecular Weight (g/mol)**	**Rotateable bonds**
Tamoxifen	-7.3, -8.6, -8.6	0	2	5.996	371.5	8
Asarinin	-9.0, -9.1, -10.2	0	6	3.2192	354.358	2

Tamoxifen, slightly basic due to the presence of amino groups, exhibits stability under standard storage conditions but may degrade when exposed to light, moisture, or elevated temperatures, necessitating proper storage and handling practices. Similarly, asarinin's moderate lipophilicity influences its absorption and distribution, with considerations for stability under various storage conditions and environmental factors.

4.22 Docking Score Comparison

Both the Tamoxifen and Asarinin were docked against three selected proteins one after other. Table 4.27 shows that the Lead Compound Asarinin has a much higher vina score than that of standard drug which is Tamoxifen. This result indicates that Asarinin can block PR receptor or bind to it more effectively as compare to Tamoxifen.

4.23 Docking Analysis Comparison

The docking results were analyzed using LigPlot based on the number of Hydrogen and Hydrophobic interactions along with number of interacting amino acids and stearic interactions. Figure 4.36 and figure 4.37 indicates the comparative analysis of interactions existing between Tamoxifen and PR along with those between

Asarinin and PR. Tamoxifen formed 12 hydrophobic interactions and no Hydrogen bond. Whereas, Asarinin formed 9 hydrophobic interactions and 2 Hydrogen bonds respectively.

Tamoxifen has also been shown to induce apoptosis in estrogen receptor-positive cells. This effect is thought to be the result of inhibition of protein kinase C, which prevents DNA synthesis. Another theory for the apoptotic effects of tamoxifen is due to an approximately 3-fold increase in intra-cellular and mitochondrial calcium ion levels after administration or induction of tumor growth factor β [59]. Figure 4.26 illustrates the molecular mechanism of action of Tamoxifen as an ER inhibitor [59].

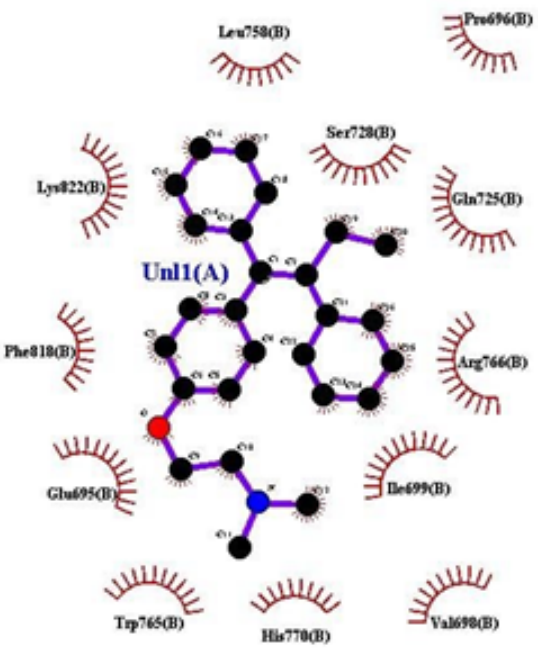


FIGURE 4.26: Interaction of Tamoxifen with PR receptor protein

Figure 4.38 shows that 2 hydrogen bonds are formed with 3.14 and 3.17 bond distances respectively.

The amino acids involved in the formation of hydrophobic interactions included: Lys769B, Val698B, Arg766B, Gly716B, Trp 732B, Leu758B,Pro696B, Glu95B and Val729B.

These amino acids play crucial roles in stabilizing the ligand-protein complex, forming a network of interactions that contribute to the overall stability and specificity of the complex.

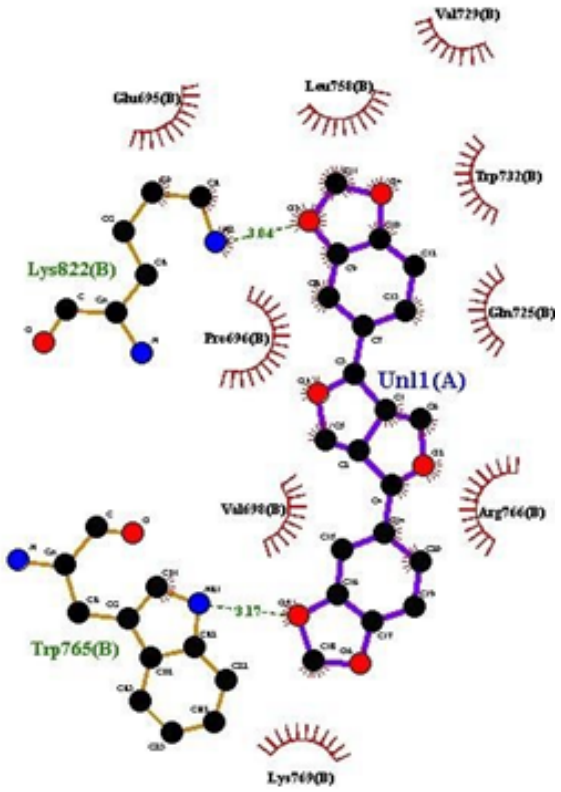


FIGURE 4.27: Interaction of Tamoxifen with PR receptor protein

Figure 4.37 illustrates the participation of specific amino acids, including Lys 322B, Phr 818 B, Glu 695 B, Trp 765 B, His 770 B, Val 698 B, Thr 699 B, Arg 766 B, Gln 725 B, Ser 728 B, Pro 696 B, and Leu 758B, in hydrophobic interactions within the binding pocket.

The presence of these amino acids indicates the formation of strong hydrophobic interactions, which are essential for maintaining the structural integrity of the ligand-protein complex. Such interactions contribute significantly to the stability and specificity of the complex, influencing its overall functionality and biological activity.

4.24 MTT Assay Analysis Results

Viable cells with active metabolism convert MTT into a purple colored formazan with an absorbance maximum near 570nm. When cells die, they lose the ability to convert MTT to formazan, thus the color formation serves as a useful and convenient marker of only the viable cells. Comparing cell viability between different concentrations and types of extracts help assess impact of these substances on the health and survival of MCF cells[34]. Positive control shows 100% cell viability as the sample was not treated with any compound. At different concentrations different cell viability percentages were seen. High cell viability in a particular condition implies that the cells are relatively unaffected or even positively influenced by the treatment while low viability may indicate cytotoxicity or inhibitory effects[60]. The percentage of viable cells is calculated using the following equation:

$$\text{Viable cells} = \frac{\text{Total number of viable cells per milliliter of aliquot}}{\text{Total number of cells per milliliter of aliquot}} \times 100.$$

At 250ppm, 80% cell viability was seen using Organic extract which is less as compare to one using aqeous extract at same concentration, 85% cell viability score. At 500 ppm, cell viability dropped to 68% using Organic extract and 75% using aqueous extract. At 1000 ppm cell viability again dropped to 45% using organic extract and 57% using aqeous extract.

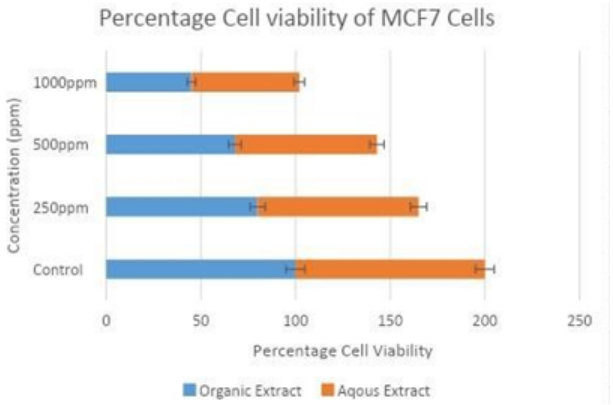


FIGURE 4.28: Percentage cell viability of MCF7 Cells

At high concentration Methanolic extract showed minimum Cell viability which indicates its inhibitory role as compared to aqeous extract as shown in Figure 4.29.

4.25 Results Using Organic Extract

At 250ppm, the organic extract resulted in 80% cell viability, indicating that at this concentration, the organic extract is relatively less toxic to MCF-7 cells. At 500ppm, the cell viability decreased to 68% suggesting increased cytotoxicity as the concentration of organic extract doubled. At 1000ppm, the cell viability dropped further to 45% , indicating a dose-dependent decrease in cell viability and increased cytotoxicity of the organic extract at higher concentrations.

4.26 Results Using Aqueous Extract

At 250ppm, the aqueous extract resulted in 85% cell viability, slightly higher than that of organic extract at the same concentration, suggesting lower toxicity. At 500ppm, the cell viability decreased to 75%, similar to the trend observed with organic extract, indicating dose-dependent decrease in cell viability. At 1000ppm, the aqueous extract showed 57% cell viability, again demonstrated a dose-dependent decrease but with higher viability compared to the organic extract at the same concentration.

Both organic and aqueous extracts of *Asarum canadense* exhibit concentration dependent cytotoxicity against MCF-7 cells. The aqueous extract generally showed slightly higher cell viability compared to the organic extract at equivalent concentrations, suggesting the potential differences in the composition and bioavailability of active compounds between the two extraction methods. However both extracts demonstrated cytotoxic effects, with higher concentrations leading to lower cell viability, indicating the potential antiproliferative properties of *Asarum canadense* extracts against MCF-7 cells.

Chapter 5

Conclusion and Future Prospects

5.1 Conclusion

The study aimed to determine the bioactive compounds in the plant *Asarum canadense* also known as Canadian wild ginger. The antitumor properties were evaluated to further investigate the plant for medicinal use as breast cancer alternative therapy. For this pupose 10 ligands were selected to be docked against important proteins involved in Breast cancer ailments.

The structure of these Ligands were easily availablein PubChem and protein structure were available at PDB. All the ligands were docked against the receptor proteins i.e., ER,PR and Ki-67 via CB Dock2. The results were visualized using Pymol and were analyzed through Ligplot. All the ligands follow lipinski's rule. Organic extract gave better inhibitory results as compare to aqueous extract.

Aqueous extract showed high cell viability making it comparatively poor inhibitor. Asarinin stood at top in producing competitive results with standard drug already been employed as compared to other selected Ligands along with few anomalous behavior trends in terms of ADMET properties.

5.2 Recommendations

As per findings of this research lead compound should be exploited more against breast cancer therapy. With these other active constituents like quercetin, myrcene, neral, asarinin, alpha terpinene, beta phallandrene, p-cymene and borneol have alse shown competitive positive results. Previously *Asarum canadense* has been used as antioxidant, anti-inflammatory and anti-viral purposes. However, its dose is of crucial significance. Many deaths are also reported due to its over dose. *Asarum canadense* should be explored more to minimize its casualties and to employ it for better treatment candidate of breast cancer patients.

Bibliography

[1] A. H. Abkar, M. S. Djati, and W. Widodo, "In silico study to predict the potential of beta-asarone, methyl piperonylketone, coumaric acid in piper crocatum as anticancer agents," *The Journal of Experimental Life Science*, vol. 11, no. 3, pp. 89–99, 2021.

[2] Z. A. Lone, Y. Lone, S. S. Khan, A. A. Wani, and M. I. Reshi, "Hepatoprotective medicinal plants used by the gond and bhill tribals of district raisen madhya pradesh, india," *Journal of Medicinal Plants Research*, vol. 9, no. 12, pp. 400–406, 2015.

[3] B. Adamczyk, M. Antczak, and M. Szachniuk, "Rnasolo: a repository of cleaned pdb-derived rna 3d structures," *Bioinformatics*, vol. 38, no. 14, pp. 3668–3670, 2022.

[4] W. F. Anderson, K. C. Chu, and S. Chang, "Inflammatory breast carcinoma and noninflammatory locally advanced breast carcinoma: distinct clinicopathologic entities?" *Journal of clinical oncology*, vol. 21, no. 12, pp. 2254–2259, 2003.

[5] G. Agarwal, P. Pradeep, V. Aggarwal, C.-H. Yip, and P. S. Cheung, "Spectrum of breast cancer in asian women," *World journal of surgery*, vol. 31, no. 5, pp. 1031–1040, 2007.

[6] L. M. Kelly, "Taxonomy of asarum section asarum (aristolochiaceae)," *Systematic Botany*, vol. 26, no. 1, pp. 17–53, 2001.

[7] M. Greenwell and P. Rahman, "Medicinal plants: their use in anticancer treatment," *International journal of pharmaceutical sciences and research*, vol. 6, no. 10, p. 4103, 2015.

[8] A. Lujambio and S. W. Lowe, "The microcosmos of cancer," *Nature*, vol. 482, no. 7385, pp. 347–355, 2012.

[9] S. Dudoit, J. Fridlyand, and T. P. Speed, "Comparison of discrimination methods for the classification of tumors using gene expression data," *Journal of the American statistical association*, vol. 97, no. 457, pp. 77–87, 2002.

[10] D. Hauner and H. Hauner, "Metabolic syndrome and breast cancer: is there a link?" *Breast care*, vol. 9, no. 4, pp. 277–281, 2014.

[11] W. F. Anderson, K. C. Chu, S. Chang, and M. E. Sherman, "Comparison of age-specific incidence rate patterns for different histopathologic types of breast carcinoma," *Cancer Epidemiology Biomarkers & Prevention*, vol. 13, no. 7, pp. 1128–1135, 2004.

[12] C. M. Perou, T. Sørlie, M. B. Eisen, M. Van De Rijn, S. S. Jeffrey, C. A. Rees, J. R. Pollack, D. T. Ross, H. Johnsen, L. A. Akslen *et al.*, "Molecular portraits of human breast tumours," *nature*, vol. 406, no. 6797, pp. 747–752, 2000.

[13] F. Cardoso, L. Cataliotti, A. Costa, S. Knox, L. Marotti, E. Rutgers, and M. Beishon, "European breast cancer conference manifesto on breast centres/units," *European Journal of Cancer*, vol. 72, pp. 244–250, 2017.

[14] H. Nagase, R. Visse, and G. Murphy, "Structure and function of matrix metalloproteinases and timps," *Cardiovascular research*, vol. 69, no. 3, pp. 562–573, 2006.

[15] Q. Yang, F. Shen, F. Zhang, X. Bai, Y. Zhang, and H. Zhang, "The combination of two natural medicines, chuanxiong and asarum: A review of the chemical constituents and pharmacological activities," *Journal of Chemical Research*, vol. 45, no. 11-12, pp. 957–976, 2021.

[16] A. Attard, M. Gourgues, E. Galiana, F. Panabieres, M. Ponchet, and H. Keller, "Strategies of attack and defense in plant–oomycete interactions, accentuated for phytophthora parasitica dastur (syn. p. nicotianae breda de haan)," *Journal of Plant Physiology*, vol. 165, no. 1, pp. 83–94, 2008.

[17] G. M. Morris and M. Lim-Wilby, "Molecular docking," *Molecular modeling of proteins*, pp. 365–382, 2008.

[18] Y. Chen and D. Zhi, "Ligand–protein inverse docking and its potential use in the computer search of protein targets of a small molecule," *Proteins: Structure, Function, and Bioinformatics*, vol. 43, no. 2, pp. 217–226, 2001.

[19] B. Quaresima, M. C. Faniello, F. Baudi, T. Crugliano, M. Di Sanzo, G. Cuda, F. Costanzo, and S. Venuta, "Missense mutations of brca1 gene affect the binding with p53 both in vitro and in vivo," *Oncology reports*, vol. 16, no. 4, pp. 811–815, 2006.

[20] S. Annunziato, M. Barazas, S. Rottenberg, and J. Jonkers, "Genetic dissection of cancer development, therapy response, and resistance in mouse models of breast cancer," in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 81. Cold Spring Harbor Laboratory Press, 2016, pp. 141–150.

[21] M. Blum, H.-Y. Chang, S. Chuguransky, T. Grego, S. Kandasaamy, A. Mitchell, G. Nuka, T. Paysan-Lafosse, M. Qureshi, S. Raj *et al.*, "The interpro protein families and domains database: 20 years on," *Nucleic acids research*, vol. 49, no. D1, pp. D344–D354, 2021.

[22] N. S. Pagadala, K. Syed, and J. Tuszynski, "Software for molecular docking: a review," *Biophysical reviews*, vol. 9, pp. 91–102, 2017.

[23] W. D. Ihlenfeldt, E. E. Bolton, and S. H. Bryant, "The pubchem chemical structure sketcher," *Journal of cheminformatics*, vol. 1, pp. 1–9, 2009.

[24] Z. Li, H. Wan, Y. Shi, and P. Ouyang, "Personal experience with four kinds of chemical structure drawing software: review on chemdraw, chemwindow,

isis/draw, and chemsketch," *Journal of chemical information and computer sciences*, vol. 44, no. 5, pp. 1886–1890, 2004.

[25] S. Sukumaran, A. Zochedh, T. M. Viswanathan, A. B. Sultan, and T. Kathiresan, "Theoretical investigation of 5-fluorouracil and tamoxifen complex–structural, spectrum, dft, admet and docking simulation," *Polycyclic Aromatic Compounds*, pp. 1–18, 2023.

[26] J. Meiler and D. Baker, "Rosettaligand: Protein–small molecule docking with full side-chain flexibility," *Proteins: Structure, Function, and Bioinformatics*, vol. 65, no. 3, pp. 538–548, 2006.

[27] P. K. Thakur and M. I. Hassan, "Discovering a potent small molecule inhibitor for gankyrin using de novo drug design approach," *International Journal of Computational Biology and Drug Design*, vol. 4, no. 4, pp. 373–386, 2011.

[28] D. Brown, T. Adams, and N. Keller, "Aspergillus has distinct fatty acid synthases for primary and secondary metabolism," *Proceedings of the National Academy of Sciences*, vol. 93, no. 25, pp. 14 873–14 877, 1996.

[29] D. T. Jones, W. R. Taylor, and J. M. Thornton, "The rapid generation of mutation data matrices from protein sequences," *Bioinformatics*, vol. 8, no. 3, pp. 275–282, 1992.

[30] S. Ekins and J. Rose, "In silico adme/tox: the state of the art," *Journal of Molecular Graphics and Modelling*, vol. 20, no. 4, pp. 305–309, 2002.

[31] S. Ekins, C. L. Waller, P. W. Swaan, G. Cruciani, S. A. Wrighton, and J. H. Wikel, "Progress in predicting human adme parameters in silico," *Journal of pharmacological and toxicological methods*, vol. 44, no. 1, pp. 251–272, 2000.

[32] G. Diaz, M. Melis, A. Musinu, M. Piludu, M. Piras, and A. Falchi, "Localization of mtt formazan in lipid droplets. an alternative hypothesis about the nature of formazan granules and aggregates," *European Journal of Histochemistry*, vol. 51, no. 3, pp. 213–218, 2007.

[33] C.-Y. Wu and L. Z. Benet, "Predicting drug disposition via application of bcs: transport/absorption/elimination interplay and development of a biopharmaceutics drug disposition classification system," *Pharmaceutical research*, vol. 22, pp. 11–23, 2005.

[34] M. Chang, "Tamoxifen resistance in breast cancer," *Biomolecules & therapeutics*, vol. 20, no. 3, p. 256, 2012.

[35] W. P. Walters, "Going further than lipinski's rule in drug design," *Expert opinion on drug discovery*, vol. 7, no. 2, pp. 99–107, 2012.

[36] Ş. Comşa, A. M. Cimpean, and M. Raica, "The story of mcf-7 breast cancer cell line: 40 years of experience in research," *Anticancer research*, vol. 35, no. 6, pp. 3147–3154, 2015.

[37] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays," *Journal of immunological methods*, vol. 65, no. 1-2, pp. 55–63, 1983.

[38] N. G. Egeland, K. Jonsdottir, K. L. Lauridsen, I. Skaland, C. F. Hjorth, E. G. Gudlaugsson, S. Hamilton-Dutoit, T. L. Lash, D. Cronin-Fenton, and E. A. Janssen, "Digital image analysis of ki-67 stained tissue microarrays and recurrence in tamoxifen-treated breast cancer patients," *Clinical Epidemiology*, pp. 771–781, 2020.

[39] S. Akhlaq, S. A. Ara, B. Ahmad, M. Fazil, U. Akram, M. Haque, and A. A. Khan, "Interventions of unani medicine for maintenance of health with special reference to air quality: An evidence-based review," *Reviews on Environmental Health*, vol. 38, no. 1, pp. 85–96, 2023.

[40] D. E. Pires, T. L. Blundell, and D. B. Ascher, "pkcsm: predicting small-molecule pharmacokinetic and toxicity properties using graph-based signatures," *Journal of medicinal chemistry*, vol. 58, no. 9, pp. 4066–4072, 2015.

[41] Y. Yeni and A. R. Rizky, "The prediction of pharmacokinetic properties of compounds in hemigraphis alternate (burm. f.) t. ander leaves using pkcsm,"

Indonesian Journal of Chemistry (Indones. J. Chem), vol. 22, no. 4, pp. 1081–1089, 2022.

[42] T. Sakurai, T. Uruno, Y. Sugiura, T. Tatsuguchi, K. Yamamura, M. Ushijima, Y. Hattori, M. Kukimoto-Niino, C. Mishima-Tsumagari, M. Watanabe *et al.*, "Cholesterol sulfate is a dock2 inhibitor that mediates tissue-specific immune evasion in the eye," *Science signaling*, vol. 11, no. 541, p. eaao4874, 2018.

[43] Y. Liu, X. Yang, J. Gan, S. Chen, Z.-X. Xiao, and Y. Cao, "Cb-dock2: Improved protein–ligand blind docking by integrating cavity detection, docking and homologous template fitting," *Nucleic acids research*, vol. 50, no. W1, pp. W159–W164, 2022.

[44] A. Sofronov, I. Nizamov, L. Almetkina, L. Nikitina, D. Fatyhova, P. Zelenikhin, O. Il'Inskaya, and R. Cherkasov, "Monoterpenoids dithiophosphates. synthesis and biological activity," *Russian journal of general chemistry*, vol. 80, pp. 1267–1271, 2010.

[45] G.-X. Liu, F. Xu, M.-Y. Shang, X. Wang, and S.-Q. Cai, "The relative content and distribution of absorbed volatile organic compounds in rats administered asari radix et rhizoma are different between powder-and decoction-treated groups," *Molecules*, vol. 25, no. 19, p. 4441, 2020.

[46] S. Björkman, "Prediction of the volume of distribution of a drug: which tissue-plasma partition coefficients are needed?" *Journal of pharmacy and pharmacology*, vol. 54, no. 9, pp. 1237–1245, 2002.

[47] M. J. Moreno, L. M. Loura, J. Martins, A. Salvador, and A. Velazquez-Campoy, "Analysis of the equilibrium distribution of ligands in heterogeneous media–approaches and pitfalls," *International Journal of Molecular Sciences*, vol. 23, no. 17, p. 9757, 2022.

[48] S. Modanwal, A. K. Maurya, S. K. Mishra, and N. Mishra, "Development of qsar model using machine learning and molecular docking study of polyphenol derivatives against obesity as pancreatic lipase inhibitor," *Journal of Biomolecular Structure and Dynamics*, vol. 41, no. 14, pp. 6569–6580, 2023.

[49] N. M. A. Ivane, S. A. Haruna, M. Zekrumah, F. K. R. Elysé, M. O. Hassan, S. B. Hashim, H. E. Tahir, and D. Zhang, "Composition, mechanisms of tingling paresthesia, and health benefits of sichuan pepper: A review of recent progress," *Trends in Food Science & Technology*, vol. 126, pp. 1–12, 2022.

[50] M. Elshal, N. Eid, I. El-Sayed, W. El-Sayed, and A. A. Al-Karmalawy, "Concanavalin-a shows synergistic cytotoxicity with tamoxifen via inducing apoptosis in estrogen receptor-positive breast cancer: In vitro and molecular docking studies," *Pharmaceutical Sciences*, vol. 28, no. 1, pp. 76–85, 2021.

[51] S. Z. Ali, Z. W. Baloch, B. Cochand-Priollet, F. C. Schmitt, P. Vielh, and P. A. VanderLaan, "The 2023 bethesda system for reporting thyroid cytopathology," *Thyroid*, vol. 33, no. 9, pp. 1039–1044, 2023.

[52] G. N. Wade and H. W. Heller, "Tamoxifen mimics the effects of estradiol on food intake, body weight, and body composition in rats," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 264, no. 6, pp. R1219–R1223, 1993.

[53] B. Ahmed, U. A. Ashfaq, M. T. ul Qamar, and M. Ahmad, "Anti-cancer potential of phytochemicals against breast cancer: Molecular docking and simulation approach," ——— *Bangladesh Journal of Pharmacology*———, vol. 9, no. 4, pp. 545–50, 2014.

[54] J. Du, W.-L. Lu, X. Ying, Y. Liu, P. Du, W. Tian, Y. Men, J. Guo, Y. Zhang, R.-J. Li *et al.*, "Dual-targeting topotecan liposomes modified with tamoxifen and wheat germ agglutinin significantly improve drug transport across the blood- brain barrier and survival of brain tumor-bearing animals," *Molecular pharmaceutics*, vol. 6, no. 3, pp. 905–917, 2009.

[55] K. Dickschen, S. Willmann, K. Thelen, J. Lippert, G. Hempel, and T. Eissing, "Physiologically based pharmacokinetic modeling of tamoxifen and its metabolites in women of different cyp2d6 phenotypes provides new insight into the tamoxifen mass balance," *Frontiers in Pharmacology*, vol. 3, p. 92, 2012.

[56] S.-L. Mo, Z.-W. Zhou, L.-P. Yang, M. Q. Wei, and S.-F. Zhou, "New insights into the structural features and functional relevance of human cytochrome p450 2c9. part i," *Current drug metabolism*, vol. 10, no. 10, pp. 1075–1126, 2009.

[57] S.-H. Jeong, J.-H. Jang, H.-Y. Cho, and Y.-B. Lee, "Simultaneous determination of asarinin, β-eudesmol, and wogonin in rats using ultraperformance liquid chromatography–tandem mass spectrometry and its application to pharmacokinetic studies following administration of standards and gumiganghwaltang," *Biomedical Chromatography*, vol. 35, no. 4, p. e5021, 2021.

[58] M. Flynn, K. A. Heale, and L. Alisaraie, "Mechanism of off-target interactions and toxicity of tamoxifen and its metabolites," *Chemical Research in Toxicology*, vol. 30, no. 7, pp. 1492–1507, 2017.

[59] A. G. Z. Matthes, G. Uemura, L. Kerr, Â. C. S. Matthes, R. A. D. Michelli, M. A. A. K. Folgueira, and R. A. da Costa Viera, "Feasibility of oncoplastic techniques in the surgical management of locally advanced breast cancer," *International Journal of Surgery*, vol. 10, no. 9, pp. 500–505, 2012.

[60] W. Y. Ho, S. K. Yeap, C. L. Ho, R. A. Rahim, and N. B. Alitheen, "Development of multicellular tumor spheroid (mcts) culture from breast cancer cell and a high throughput screening method using the mtt assay," 2012.