ELUCIDATION OF NATURAL COMPOUNDS INTERFERING WITH HASHIMOTO'S THYROIDITIS MEDIATED INDUCTION OF BREAST CANCER

A DISSERTATION

SUBMITTED IN THE PARTIAL FULFILMENT OF THE

REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

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CANDIDATE' DECLARATION

I, Shayon Mahalanobis, Roll No., 2K20/MSCBIO/29, student of M.Sc. Biotechnology, hereby declare that the dissertation project titled "**Elucidation of natural compounds interfering with Hashimoto's thyroiditis mediated induction of breast cancer**" which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirements for the award of the degree of Master of Science, is original and not derived from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

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CERTIFICATE

I hereby certify that the dissertation project titled "**Elucidation of natural compounds interfering with Hashimoto's thyroiditis mediated induction of breast cancer**" which is submitted by Shayon Mahalanobis, 2K20/MSCBIO/29, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of requirement for the award of the degree of Master of Science, is a record of work carried out by the student under my supervision. To the best of my knowledge, this work has not been submitted in part of any Degree or Diploma to this University or elsewhere.

PLACE: Delhi **DR. ASMITA DAS**

DATE: **SUPERVISOR**

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I, Shayon Mahalanobis, feel proud to present to present the dissertation project as a part of curriculum of M.Sc. Biotechnology. This project could not have been completed without the support and guidance of all the people who assisted in the completion of the project.

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ABSTRACT

Breast cancer accounts up to 25% of cases of worldwide cancers and there exist several causes which lead to manifestation of breast cancer. Women suffering from thyroid autoimmunity, particularly Hashimoto's thyroiditis, have a greater propensity to develop breast cancer, of the ER positive subtype. Hashimoto's thyroiditis causes hypothyroidism, which increases levels of free estradiol in the body and increased free estradiol is correlated with greater chance of developing breast cancer. Several FDA approved drugs, which are synthetic formulations, are approved for treatment of breast cancer, but there are observed side effects including development of autoimmune disorders. The risk of developing multiple autoimmune syndrome in patients already suffering from Hashimoto's thyroiditis is too great. Thus, natural compounds are needed as inhibitors of various pathways of breast cancer. Pharmacophore modelling of FDA approved drugs was performed to obtain natural compounds with similar structures and docking analysis was done against the target receptors. Anisotine obtained from *Justicia adhatoda* was observed to be the lead compound in this study. *Justicia adhatoda*, also known as Malabar nut is being used in Indian traditional medicine for thousands of years, so anisotine can most likely be well tolerated by the body without any side effects and be used as a possible replacement for several drugs for treating patients of breast cancer already suffering from Hashimoto's thyroiditis.

Keywords: Hashimoto's thyroiditis, Breast cancer, ER positive, Aromatase, CDK6, DHFR, Docking, Bioavailability testing, Natural compound, Anisotine

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
CDK6	Cyclin Dependent Kinase 6
DHFR	Dihydrofolate Reductase
E2	17 β-estradiol
ER	Estrogen Receptor
FDA	Food and Drug Administration
GD	Graves' Disease
HT	Hashimoto's Thyroiditis
IBIS-II	International Breast Cancer Intervention Study II
IMPPAT	Indian Medicinal Plants, Phytochemistry and Therapeutics
MAS	Multiple autoimmune syndrome
NK-1R	Neurokinin-1 Receptor
PDB	Protein Data Bank
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol-3-kinase
PLC	Phospholipase C
Rb	Retinoblastoma
SCLE	Subacute cutaneous lupus erythematosus
SHBG	Sex hormone binding globulin
T3	Triiodothyronine

T4	Thyroxine
TPOAb	Anti-thyroperoxidase antibody
TSH	Thyroid stimulating hormone

CHAPTER 1
INTRODUCTION

Thyroid gland is required for several metabolic reactions. Thyroid autoimmunity is a condition where the immune cells of the host attack the thyroid glands interpreting it as foreign to the host. This attack results in damage to the thyroid gland and causes its function to diminish over time. Thyroid autoimmunity is a condition which lasts for a lifetime once it manifests. Thyroid gland controls and coordinates metabolic homoeostasis and any disorder disrupts this careful balance[1]. Thyroid autoimmunity manifests itself in two forms: hypothyroidism and hyperthyroidism. The occurrence and manifestation of either of the two types depends on the type of damage to the thyroid gland and other related conditions. There is a transcription factor NF-κB which is directly related to autoimmunity [2]. It is involved in controlling and regulating of genes to maintain immunity, inflammation, cell survival, cell proliferation, and cell differentiation.

1.1. Hashimoto's Thyroiditis

Hashimoto's thyroiditis(HT) is an autoimmune condition which leads to hypothyroidism. This disease is more common in women aged from 30 to 50. Women have a greater propensity of developing HT about 4-10 times greater than that of men[3].When thyroid gland is infiltrated by lymphocytes, they come in close contact to thyrocytes, penetrate in the cytoplasm and destroy the thyrocytes[4], followed by damage to the thyroid resulting in insufficient hormone formation leading to increased concentration of Thyroid Stimulating Hormone (TSH) and lowered levels of Thyroxine(T4)[5]. Lowered levels of T4 lead to lower levels of Triiodothyronine(T3) as T4 is converted to T3 . Epidemiological studies suggest that iodine-sufficient people

are more prone to developing HT than iodine-deficient ones[3]. Complications such as diabetes, high blood pressure, and heart failure are observed. The symptoms include muscle pain, weight gain, goiter, irregular menstrual periods, and slow heart rate. Hepatitis C virus can lead to Hashimoto's disease, and other causes include genes, medicines for treating bipolar disorders, and toxin exposure[3]. The patients are diagnosed via blood tests, and levothyroxine is used to treat hypothyroidism[6].

1.2. Graves' Disease

Graves' disease(GD) is a condition with autoimmunity leading to hyperthyroidism. This disease manifests as production of antibodies, i.e., thyroid-stimulating immunoglobulins, which bind to thyroid cells and causing excessive production of thyroxine leading to GD. Immunocompromised people and women are mainly affected by this disorder and it occurs mainly in the range of 30-60 years[7]. Patients with rheumatoid arthritis, vitiligo, celiac disease and Addison's disease are likely to have GD. The complications linked with GD include irregular heartbeat, menstrual problems, osteoporosis or bone thinning, and infection in the eye, known as Graves' ophthalmopathy[7]. It can also create a problem at the time of pregnancy for both mother and child. Weight loss, trembling hands, goiter, diarrhoea, muscle weakness are some of the more common symptoms observed. The patient is known to have Graves' dermopathy, i.e., reddish thickening of skin on shins[7]. The Human T-Lymphotropic virus is a known cause in 6-8% of cases[8]. The diagnosis is done via blood tests and radioiodine therapy treats the disease[9]. Beta-blockers and anti-thyroid medicines are given to treat hyperthyroidism[7][10], and are known to cause liver failure and allergic reactions and may require thyroid surgery[11].

1.3. Breast Cancer

Breast cancer is the second most common cancer among women, which affects millions worldwide and over 25% of cases every year are breast cancer cases[12]. Women have a propensity of developing breast cancer over 100 times greater than men. Breast cancer affects women irrespective of their reproductive status with an almost similar intensity for women before and after menopause. Causes can include

anything from aging, genetic predisposition, autoimmunity, estrogen levels, sex and family history[13].

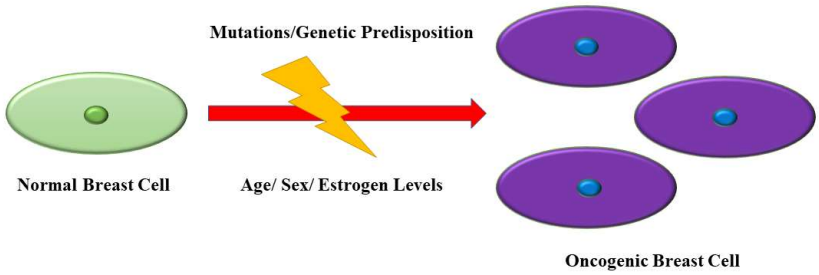


Fig 1.1: Development of Breast Cancer

Treatment of breast cancer

Treatment of breast cancer can include surgical resection of the tumor, or ovaries in case of ER positive tumor, using chemotherapy, inhibitors for pathways involved in cell cycle and radiotherapy where resection is not possible in advanced metastatic tumors[12].

In case of pathway inhibitors, is has been observed that the inhibitors often lead to the development of autoimmune disorders[14]–[17]. This raises significant concern over the use of these drugs in patients already suffering from autoimmune disorders such as Hashimoto's thyroiditis or systematic lupus erythematosus. Thus, this study aims to find natural compounds which can be used as a suitable replacement for them. Natural compounds are obtained from medicinal plants which have been used since time immemorial in Indian medicinal formulations and have a greater tolerance in comparison to the synthetic formulations used as drugs.

CHAPTER 2
LITERATURE REVIEW

2.1. Breast cancer in patients with Hashimoto's Thyroiditis

There are several clinical studies that correlate thyroid autoimmunity as a significant cause of breast cancer[18][19]. Anti-thyroid peroxidase antibody (TPOAb) has a greater propensity of causing breast cancer compared to antithyroglobulin antibody (TgAb)[20]. Autoimmunity is proven as to be a significant prognosis factor for breast cancer[18], with it being counted on a negative scale, with patients suffering from thyroid autoimmunity having a poor prognosis. It is proposed that thyroperoxidase is not fully transcribed in less differentiated breast cancer[21].

Of all the subtypes of breast cancer, in an analysis of 867 patients, Chiappa et al.[22] found ER levels to be elevated in patients, indicating that the patients of breast cancer suffering from Hashimoto's Thyroiditis, are ER positive(98%), and no other receptors showed any statistical difference between the control group and test group.

In addition to this, high estrogen levels are known to be correlated with higher chance of autoimmune response in women and the same can be extended towards the development of Hashimoto's Thyroiditis in women[23]. women with hypothyroidism have been observed to have an increase in unbound or free estrogen levels[24] and free estrogen levels are a prognostic factor of developing breast cancer in both pre- and post-menopausal women[23][24]. Elevation in free estrogen is attributed to the decrease of sex-hormone binding globulin(SHBG) , which is involved in metabolic clearance[24] of free estrogen from the body.

2.2. Factors affecting ER-positive breast cancer

2.2.1. Aromatase

Aromatase is the rate- limiting enzyme which converts testosterone to estrogen, specifically 17 β-estradiol(E2), the biologically active estrogen[27].

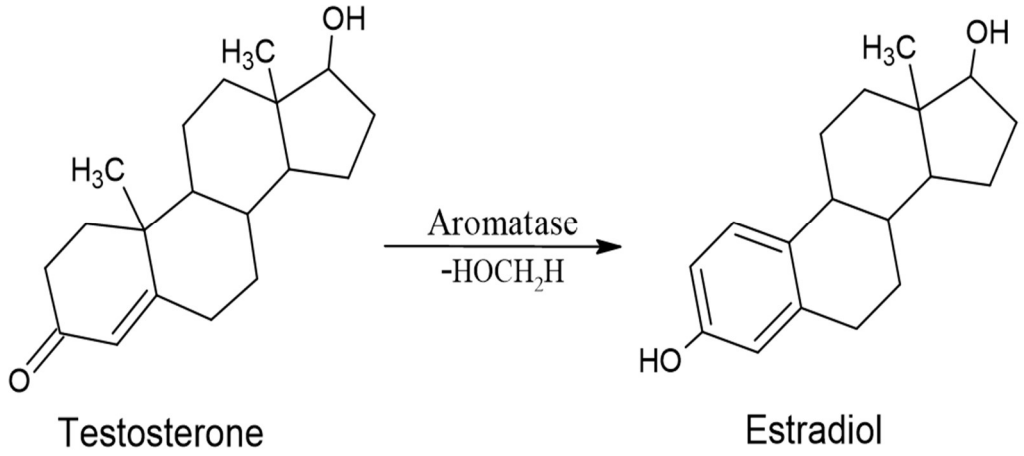


Fig 2.1: Conversion of testosterone to estradiol

Aromatase is encoded by the gene *CYP19A1*, located on chromosome 15q21.1[28] and is a member of the cytochrome p450 superfamily. Estrogen synthesised by aromatase is utilized in a myriad of functions: the most well-known function is its role in reproductive health as a hormone, but is also involved in vascular biology, metabolism, mineralization of bone and cognitive function[29].

Structure of Aromatase

Aromatase is comprised of a single chain of 503 amino acid residues, and is associated with a heme group[30].

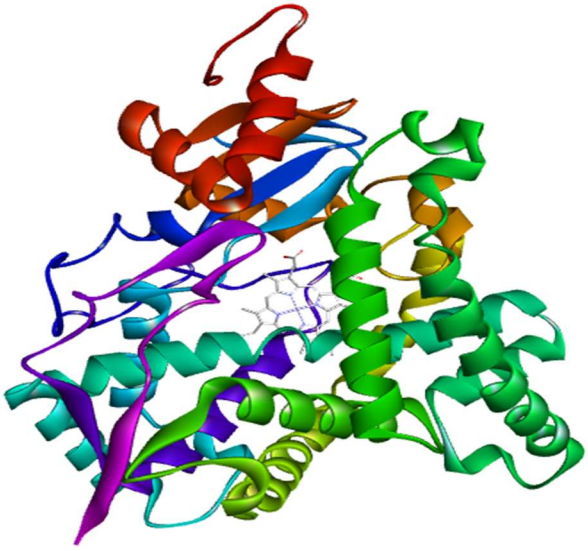


Fig 2.2: Structure of aromatase complexed with heme[30]

Role of aromatase in breast cancer

In ER positive breast cancer, aromatase plays a critical role i.e., it causes conversion of testosterone to estrogen. Three sources of aromatase production are known to support breast cancer cell development: ovaries, subcutaneous adipose tissue and the cancer cell themselves. Of them, ovaries are more common source for pre-menopausal women, while adipose tissue and breast cancer cells are the source for aromatase in post-menopausal women[31].

During normal growth and development of breast tissue, aromatase synthesis is low and uses the distal promoter 1.4, which constitutively maintains a low but constant level of aromatase production. Once cancer manifests in breast tissue, the altered cells start expressing prostaglandin E2(PGE2), which increases aromatase production by switching to promoter 1.3 and 1.7 is addition to promoter 1.4, causing a massive increase in aromatase production and by extension increase in the estrogen synthesis in the body[29] and estrogen is known to increase cell proliferation and decrease apoptosis[32].

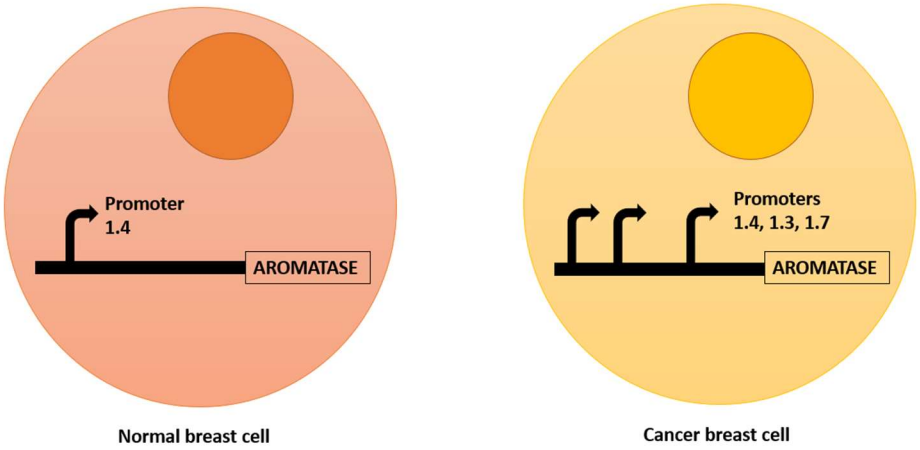


Fig 2.3: Aromatase production in normal and cancer cell

Inhibitors of aromatase

Aromatase inhibitors, are non-steroidal compounds, which bind to the aromatase and prevent binding of testosterone, which doesn't allow the conversion of testosterone to estrogen, thus limiting its synthesis. Clinical trials have shown the effectivity of anastrozole, which reduced the rate of development of breast cancer: MAP.3 and IBIS-II in the initial 5 years after the administration[33]. The updated trials for IBIS-II revealed the rate of cancer inhibition by anastrozole was maintained up to 12 years in the next follow-up[33].

Despite the benefits provided by aromatase inhibitors, they are not without side effects of their own. There are recorded evidences of anastrozole being used for ER positive breast cancer, leading to development of further autoimmune disorders such as subacute cutaneous lupus erythematosus(SCLE)[17], pruritus[14], and cutaneous vasculitis[34].

2.2.2. CDK6

Cyclin dependant kinase-6 or CDK6 is a checkpoint regulator of the cell cycle and regulates the G1/S-phase transition in complex with CDK4. It is encoded by the gene *CDK6,* located on chromosome 7q21.2. CDK6 is associated with cyclin D1 and complexes with it to phosphorylate retinoblastoma protein Rb, which releases E2F, to initiate cell cycle progression[35].

Structure of CDK6

CDK6 has a protein structure comprising of 326 amino acids and forms 5 stranded β-sheet at the amino terminal, with the carboxy terminal consisting of α-helices[36].

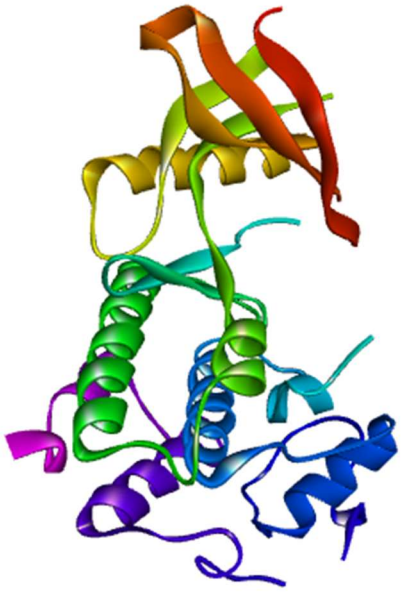


Fig 2.4: Structure of CDK6[37]

Role of CDK6 in breast cancer

CDK6 is involved in phosphorylation of Rb protein, which is utilised in cell cycle to pass through the G_1 to S transition stage and move to S-phase. In breast cancer, the cell cycle checkpoints are dysregulated and cyclin D1 production is upregulated[35]. Upregulation of *CCND1* gene, responsible for cyclin D1 production, was observed in 60% of ER positive breast cancer cases[38].

CDK6 amplification is a driver of oncogenesis in breast cancer[39] and also known to increase expression of VEGF-A and induce neoangiogenesis, promoting cancer cell proliferation[40]. CDK6 also induces drug resistance against its inhibitors and also against inhibitors of ER, as demonstrated experimentally by Yang et al.[41].

Inhibitors of CDK6

CDK6 inhibitors act on CDK6 and prevent formation of complex between cyclin D1 and CDK6. Palbociclib, ribociclib and abemaciclib are the FDA approved drugs

against CDK6[39]. Abemaciclib is the most widely used inhibitor of the three, and has potent single agent activity, with a greater potential to penetrate breast tissue compared to other inhibitors due to increased lipophilicity[42] and increasing disease free survival to >25 months when used in conjunction with aromatase inhibitors[40].

Overdependence on CDK6 inhibitors have proven to be deleterious as it can trigger resistance against itself in the body on the patient[40] and is can also trigger autoimmune disorders such as SCLE[16], where discontinuation of abemaciclib resolved the symptoms which warrants the need to carefully monitor its effects.

2.2.3. DHFR

Dihydrofolate reductase(DHFR) converts dihydrofolic acid to tetrahydrofolic acid, which is required in the synthesis of purines for cell replication. Dihydrofolate reductase is encoded by the *DHFR* gene, located on chromosome 5q14.1 region.

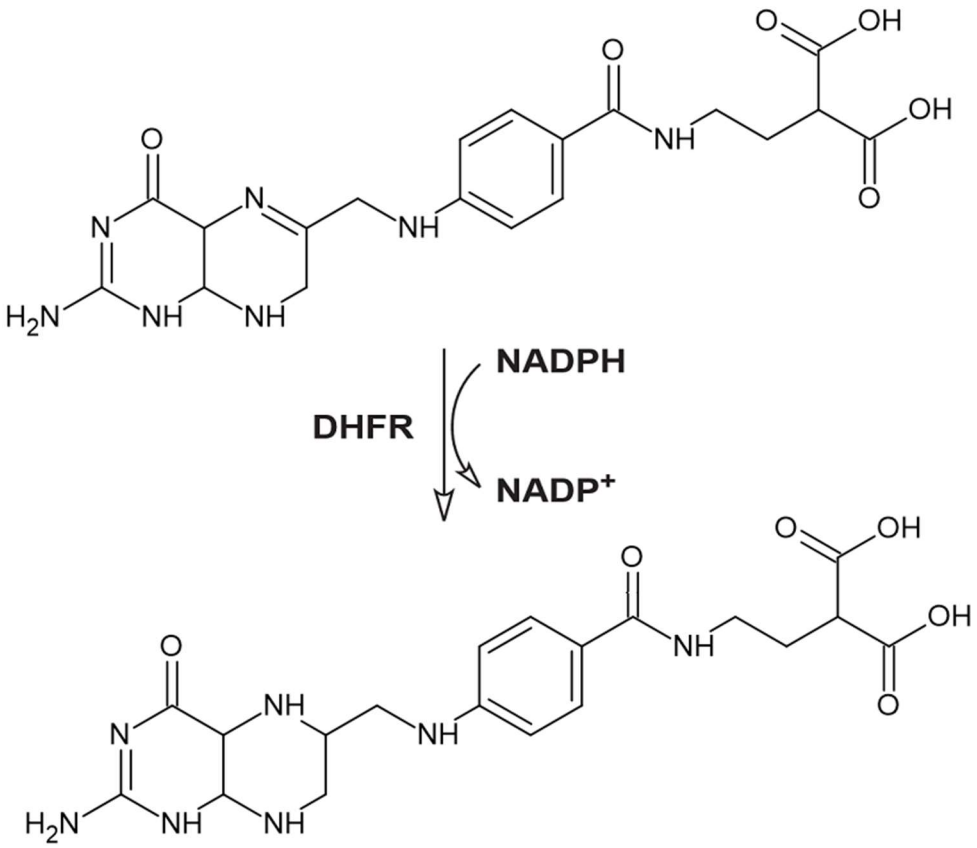


Fig 2.5: Conversion of dihydrofolate to tetrahydrofolate by DHFR

Structure of DHFR

DHFR consists of 8 stranded β-pleated sheets as the central backbone. Seven of these sheets run parallel, while the last runs antiparallel in orientation[43]. Loop1 or Met20 is a critical component of the active site, as it surrounds the subdomain near the active site[44].

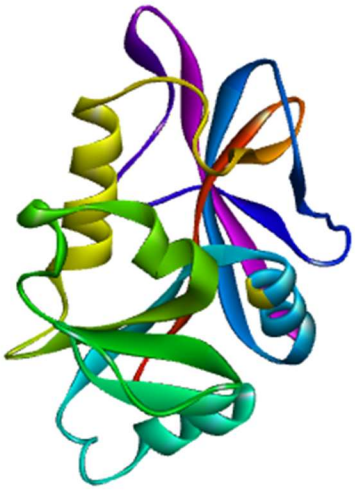


Fig 2.6: Structure of DHFR[45]

Role of DHFR in breast cancer

DHFR is observed to play a role in synthesis of purines and thus involved in cellular proliferation. In breast cancer cells, upregulation of DHFR is observed[46], with resistance towards drugs due to mutations in *DHFR* gene. Estrogen levels are also known to increase the DHFR levels and resistance towards DHFR inhibitors[46][47].

Inhibitors of DHFR

Inhibitors of DHFR, such as methotrexate are used to as antagonists of DHFR, to halt the production of tetrahydrofolate and by extension purine synthesis. Purine synthesis is a necessary step for cellular replication and halting this step inhibits replication during the S phase[48]. Methotrexate is used for invasive breast cancer and despite not having a good solubility and non-specific targeting[49], is the preferred drug of choice for inhibiting DHFR, in combination with 5-FU.

Side effects of methotrexate use include suppressed immune function, hepatotoxicity[50] and stomatitis[51], with discontinuation resolving the symptoms.

2.2.4. PI3K

Phosphatidylinositol-3-kinase(PI3K) is an enzyme responsible for converting phosphatidyl-(4,5)-bisphosphate(PIP_2) to phosphatidyl-(3,4,5)trisphosphate(PIP_3) [52]. The enzyme works in concert with extracellular signalling via G-protein coupled receptors(GPCR) to convert PIP_2 to PIP_3, which is involved in recruiting protein kinase B(AKT), which further phosphorylates mTOR to activate it, targeting other target proteins in the nucleus, triggering cellular growth and proliferation[52].

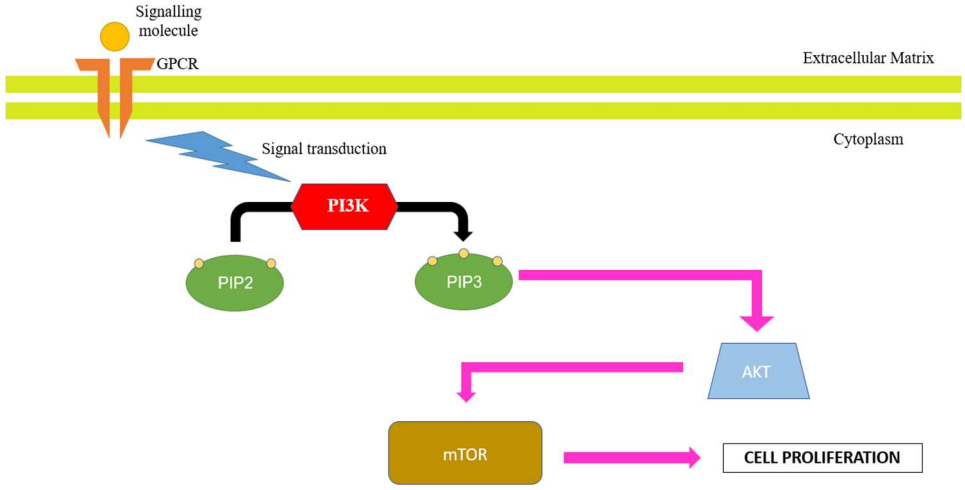


Fig 2.7: Conversion of PIP_2 to PIP_3 by PI3K leading to cellular proliferation

Structure of PI3K

PI3K comprises of 2 subunits, 85 kDa regulatory subunit, p85 and a catalytic subunit of 110 kDa, p110[53], which has 4 isoforms α, β, γ, δ of which α is the most common subunit[54].

Fig 2.8: Structure of PI3K[55]

Role of PI3K in breast cancer

PI3K, being involved in cellular proliferation, is deregulated in breast cancer, with ER positive cancers showing a mutation in α isoform of p110, encoded by *PIK3CA* gene, in about 40% of ER positive cancers[54], with advanced cancers having *PIK3CA* mutations in 30% cases[53]. Also, it is observed, that ER and PI3K pathways are correlated, with ER+ and *PIK3CA* mutations being resistant to anti-estrogen medications[56].

Inhibitors of PI3K

PI3K isoform specific inhibitors are utilised as treatment methods to stop the PI3K induced cellular signalling. Alpelisib is used as the inhibitor of p110α, which is the most common mutation of p110 subunits[56], and has shown promising results when used in addition to endocrine therapy for metastatic ER positive breast cancer[57]. Alpelisib increased the progressive free survival to 11 months from 5.7 months in case of *PIK3CA* mutated breast cancer and thus was approved by FDA in 2019[58].

Side effects associated with alpelisib include rash, dermatitis, diarrhoea, fatigue, hyperglycemia and nausea[58][59], with symptoms being resolved by discontinuation of the drugs, as they have a short half-life[56]

2.2.5 NK-1R

Neurokinins, also known as tachykinins, are neurotransmitters with a common structure of Phe-X-Gly-Met-Leu-NH_2. Three neurokinins are known: Substance P, Neurokinin A and Neurokinin B[60].

Each neurokinin has a particular receptor[60]:

- Substance P- Neurokinin-1 Receptor (NK-1R)
- Neurokinin A- Neurokinin-2 Receptor (NK-2R)
- Neurokinin B- Neurokinin-3 Receptor (NK-3R)

NK-1R is the most common receptor and a seven transmembrane GPCR and is involved in stimulus for noxious sensations and thus controls sensations of nausea and emesis[61]. In addition to this, Substance P binding causes activation and release of Phospholipase-C(PLC), which leads to downstream signalling by activation of Phospholipase A2, arachidonic acid, and leukotrienes. All these molecules result in inflammation, anti-apoptosis and cell proliferation. Thus, NK-1R is upregulated in organs affected by inflammation and in patients with inflammatory bowel disease[62]. In addition to this, NK-1R/Substance P is also known to cause other disorders like cancer and Systematic Lupus Erythematosus(SLE), Rheumatoid Arthritis(RA) and Alopecia Areata(AA)[62].

Structure of NK-1R

Neurokinin-1 receptor is a receptor of 407 residues, with high affinity binding for Substance-P. the structure shows high similarity between species, showing 94.5% similarity between humans and mice[62]. A truncated variant also occurs in humans due to incomplete splicing leading to a COOH terminal truncated protein of 311 residues[63].

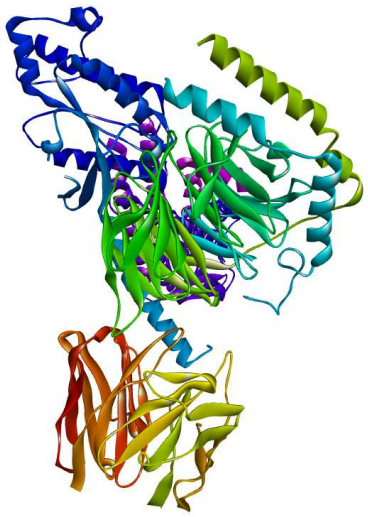


Fig 2.9: Structure of Neurokinin-1 receptor[64]

Role of NK-1R

NK-1R is involved in inflammation and thus by extension, plays a role in pathogenesis of cancer. Experimental evidences support this observation, demonstrating the fact that NK-1R and Substance P complex is involved in the proliferation of several cell lines: melanocytes, breast cancer, astrocytes, pancreatic cancer, glioblastoma, retinoblastoma etc[65]. Patients with autoimmune diseases have a higher tendency to develop

NK-1R maintains the tumor microenvironment in breast cancer in the following ways:

- Angiogenesis

  Angiogenesis is the process of formation of new blood vessels and a crucial step for cancer cell proliferation. In cancer, neoangiogenesis is a hallmark of tumor development and elevated levels of NK-1R and Substance P[66] are observed, which influences neoangiogenesis by allowing tumoral blood flow and stromal development[66].
- Metastasis

  Metastasis is the process of a benign tumor becoming metastatic i.e., the cells begin to slough off the initial tumor site and start migrating to other locations and get deposited there to form a neoplasm at the site of deposition, spreading

the tumor to different sites of the body. Substance P and NK-1R are known to cause cancer cell migration in a similar manner to leukocyte extravasation via directed chemotactic migration[65][67].

Inhibitors of Neurokinin-1 Receptor

Aprepitant is an FDA approved inhibitor of NK-1R, sold under the brand name Emend, and is an antagonist against NK-1R. It binds to the active site and prevents the interaction of NK-1R with Substance P. Aprepitant is known to be used in several diseases such as pruritus[68] and after chemotherapy induced vomiting as an antiemetic[69]. Also, it is proven as a novel inhibitor for several types of cancer including but not limited to breast cancer[70][71].

CHAPTER 3
METHODOLOGY

3.1. Identification of FDA approved drugs

FDA approved drugs for breast cancer was identified from NCI website (https://www.cancer.gov/about-cancer/treatment/drugs/breast). The drugs were downloaded in .3d sdf format from PubChem, converted to both .mol2 and .pdb formats using Biovia DS Visualizer 2021.

3.2. Pharmacophore Modelling

The drugs in .mol2 format were loaded on PharmaGist[72], a ligand based detection webserver for pharmacophore modelling. The webserver creates a pharmacophore of the input ligands and checks them over for different parameters such as spatial features, number of aromatic centres, donors, acceptors etc.

3.3. Natural Compound Selection

Once the pharmacophore was obtained, it was run on ZincPharmer[73], a webserver based search engine, which scans the entire Zinc Database[74] for structures similar to the loaded pharmacophore. The results gave the hit compound, **ZINC68198368**(Fig 3.1).

In order to identify natural compounds with structural similarity for docking purpose, the SMILES structure of **ZINC68198368** was uploaded into the IMPPAT database[75]. IMPPAT database is a curated database of Indian medicinal plants, with

the phytochemical information of each curated plant. The resulting compounds obtained were downloaded in .pdb format for docking analysis.

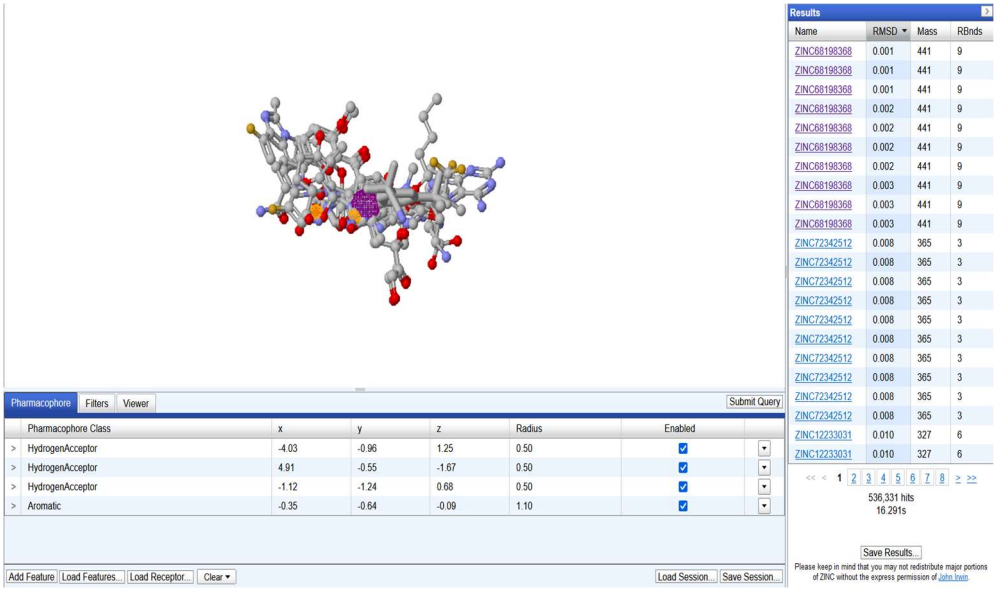


Fig 3.1 :ZincPharmer showing pharmacophore and top hit ZINC68198368

3.4. Protein Preparation

All receptors for FDA approved drugs were downloaded, in 3D conformation from PDB[76] ,complexed to their inhibitors. Heteroatoms such as water and inhibitor complex were removed using Biovia DS Visualizer 2021, with the binding coordinates of the inhibitor being recorded. Once the heteroatoms were removed, the structure was energy minimised using Swiss PDB Viewer. Energy minimisation is an important step for ensuring proper molecular arrangement.

TABLE 3.1: Receptor/inhibitor complexes taken from PDB

Receptor/Inhibitor Complex	RCSB PDB ID	Reference
Aromatase/Exemestane	3S7S	[30]
CDK6/ [5-[4-(dimethylamino)piperidin-1-yl]-1H-imidazo[4,5-b]pyridin-2-yl]-(2-isoquinolin-4-ylpyridin-4-yl)methanone	4EZ5	[37]

DHFR/ (2R)-6-[[methyl-(3,4,5-trimethoxyphenyl)amino]methyl]-1,2,5,6,7,8-hexahydroquinazoline-2,4-diamine	1S3U	[45]
PI3K/ GDC-0980	3TL5	[55]
NK-1R/Aprepitant	6HLO	[64]

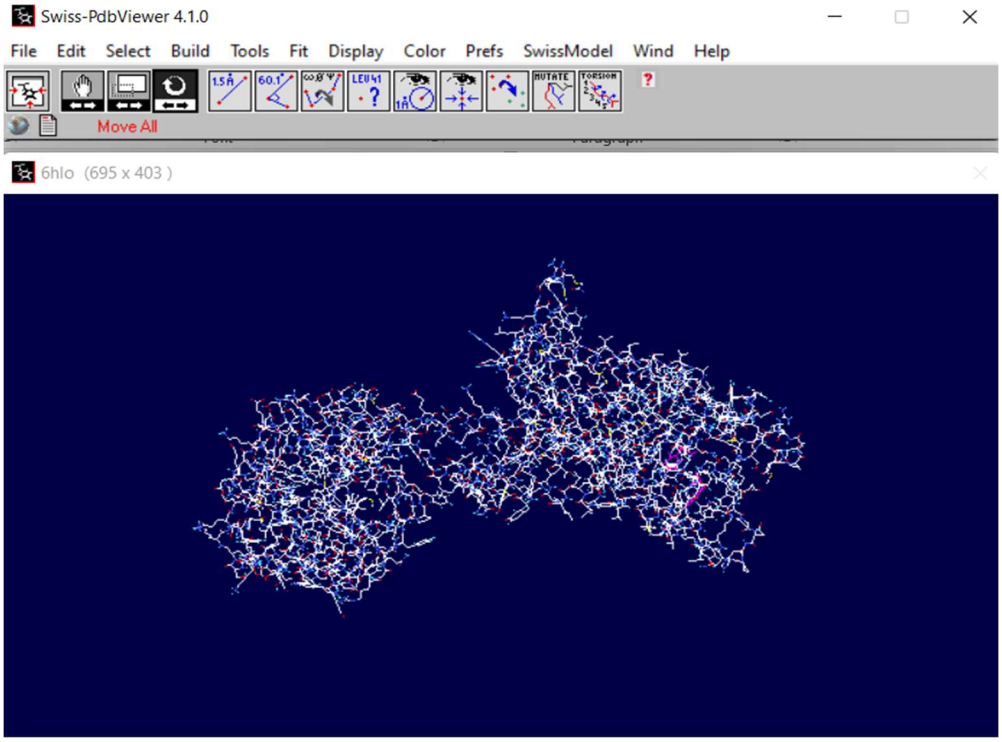


Fig 3.2: Energy minimizing using Swiss PDB Viewer

3.5. Bioavailability Testing

The natural compounds obtained were subject to bioavailability testing using SwissADME[77], on several parameters such as molecular weight, Lipinski rule of five-which determines whether the target compound can act as a drug candidate or not [78], solubility, synthetic accessibility and lead likeness. SMILES of the compounds are accessed as input. The compounds satisfying these criteria can act as lead compounds and were subjected to molecular docking.

3.6. Docking Analysis

Compounds satisfying bioavailability testing were subjected to molecular docking. For docking AutoDock ver4.2 was used. The graphical support for the same was provided by AutoDockTools ver1.5.7. Following steps were performed for docking analysis:

- The energy minimised protein structure was loaded in pdb format and polar hydrogens were added, and non-polar hydrogens were merged. This was followed by addition of Kollman charges. Kollman charges ensure correct electrostatic potential of the protein structure.

- Ligand was added in pdb format and similar process was followed as that of protein, for hydrogens and Kollman charges. In addition, Gasteiger charges were also added for the ligand.

- Grid parameters were set using the coordinates of the inhibitor obtained, with a 40x40x40 grid box to ensure the ligand is docked at the same place to ensure optimal results.

- AutoGrid was used to set the grid affinity and Lamarckian GA was used as search parameter.

- AutoDock was initiated and the different conformations were tested for binding energy between the ligand and protein. The conformation which exhibited the lowest (more negative) binding energy was selected for visualization in Biovia DS Visualizer 2021

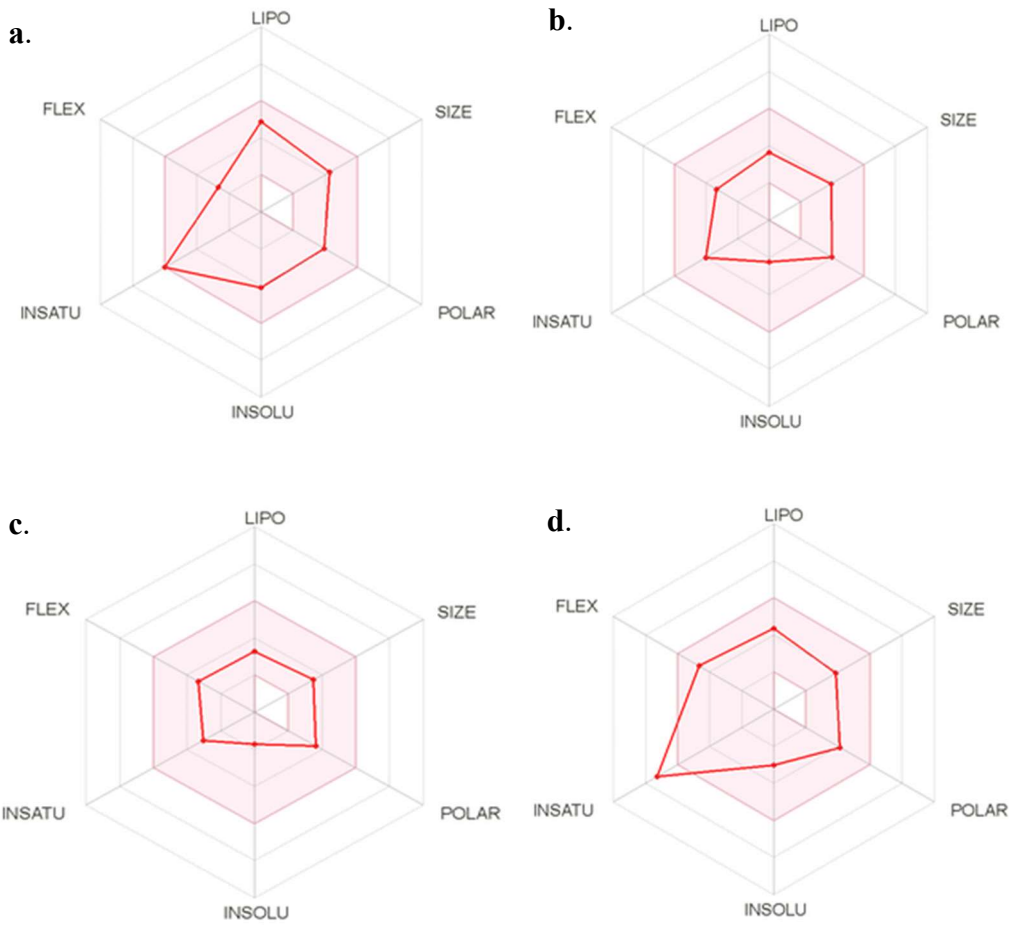
- The same steps were repeated for all the ligands and receptors.

CHAPTER 4

RESULTS

4.1. Bioavailability Testing

The natural compounds obtained from IMPPAT database were subjected to bioavailability testing to identify lead compounds. Of 35 compounds tested(Appendix A, Table S1), 6 showed zero lead likeness violations: Anisotine, Eseramine, Etamiphylline, Moupinamide, N-cis-ferulotyramine and Petasiphinol. These compounds were further subjected to docking analysis to check for binding efficiency.



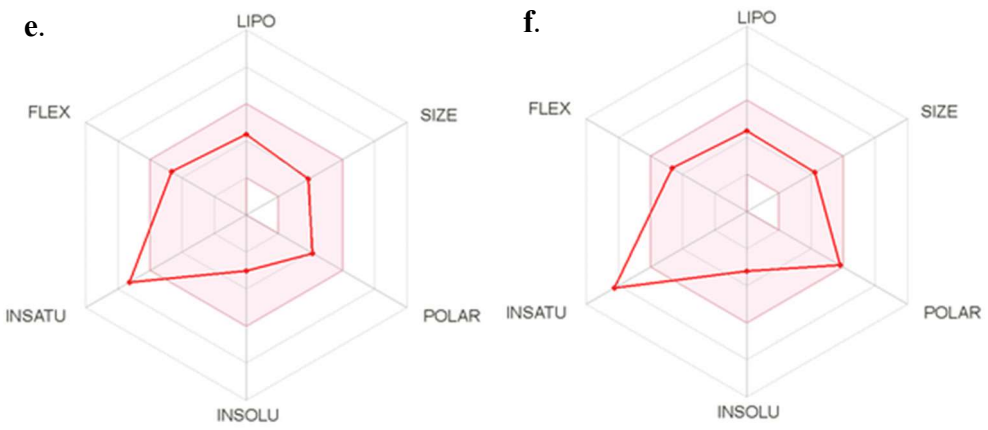


Fig 4.1: Natural compounds satisfying bioavailability parameters

a)Anisotine b)Eseramine c)Etamiphylline

d)Moupinamide e) N-cis-ferulotyramine f)Petasiphinol

4.2. Docking Studies

Docking analysis was performed for the natural compounds obtained from the IMPPAT database., FDA approved drugs were themselves used as reference ligands against which the natural compounds were analysed for lower binding energy.

4.2.1 Aromatase

TABLE 4.1: Binding Energy of compounds against Aromatase

Name of Compound	Binding Energy(kcal/mol)	Inhibition Constant(µmol)	Intermolecular Energy(kcal/mol)
Anastrozole*	-7.65	3.68	-8.61
Anisotine	-8.30	0.824	-9.49
Eseramine	-7.06	12.80	-7.27
Etamiphylline	-5.85	54.24	-7.31
Moupinamide	-7.31	5.11	-9.61
N-cis-ferulotyramine	-7.68	145.26	-7.62
Petasiphinol	-5.38	113.13	-8.67

*** FDA Approved Drug**

Anisotine and N-cis-ferulotyramine have more binding affinity towards aromatase than the approved FDA drug, anastrozole.

Anisotine shows the greatest binding efficiency among the compounds(-8.30 kcal/mol). It forms van der Waals interactions with 9 amino acids, alkyl bonds with 5 amino acids: Ile132, Phe134, Phe148, Ala306 and Met374.

In addition, it also forms two conventional hydrogen bonds with Arg115 and Ala438, and a pi-sigma bond at Ile133.

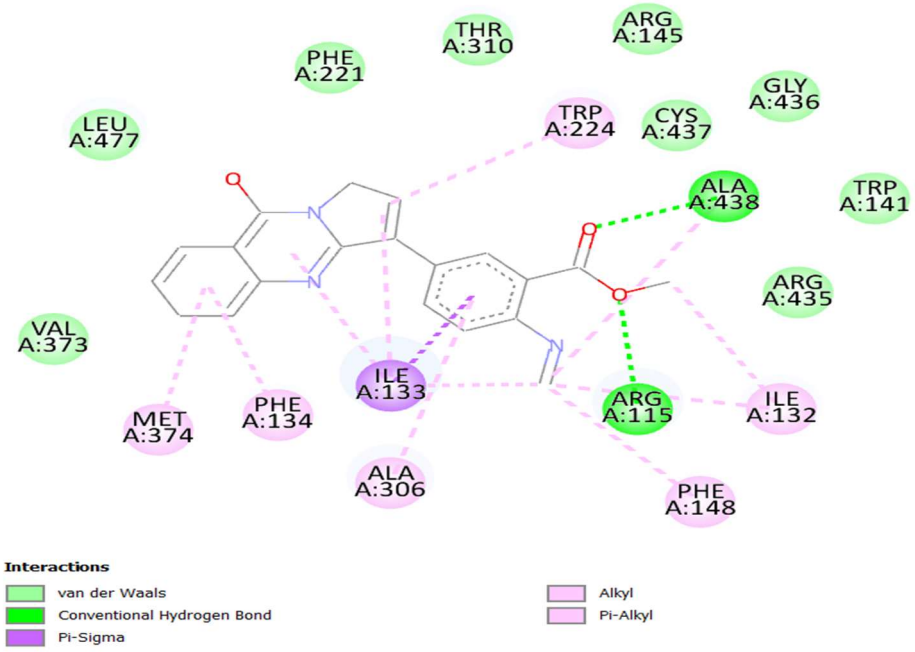


Fig 4.2: Interaction between aromatase and anisotine

N-cis-ferulotyramine shows a binding energy of -7.68 kcal/mol forms 13 van der Waals interactions, two conventional hydrogen bonds at Met374 and Leu477.

Phe221 and Cys437 form alkyl linkages with the compound and Val370, Val 373 and Leu477 form pi-alkyl bonds.

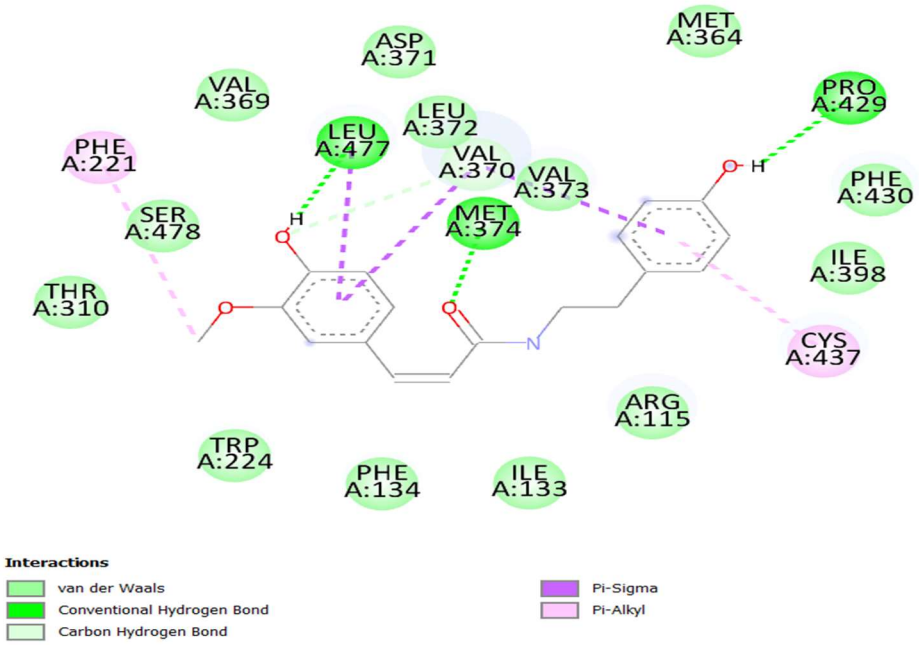


Fig 4.3: Interaction between aromatase and N-cis-ferulotyramine

4.2.2. CDK6

TABLE 4.2: Binding Energy of compounds against CDK6

Name of Compound	Binding Energy(kcal/mol)	Inhibition Constant(µmol)	Intermolecular Energy(kcal/mol)
Abemaciclib*	-9.51	0.106	-11.60
Anisotine	-9.02	0.245	-10.21
Eseramine	-7.43	3.61	-8.02
Etamiphylline	-5.27	137.15	-6.76
Moupinamide	-7.48	3.28	-9.87
N-cis-ferulotyramine	-6.70	12.27	-9.09
Petasiphinol	-7.25	4.87	-10.53

***FDA Approved Drug**

No compounds showed greater binding energy than the FDA approved drug, Abemaciclib.

4.2.3. PI3K

TABLE 4.3: Binding Energy of compounds against PI3K

Name of Compound	Binding Energy(kcal/mol)	Inhibition Constant(µmol)	Intermolecular Energy(kcal/mol)
Alpelisib*	-9.18	0.188	-10.67
Anisotine	-9.56	0.097	-10.76
Eseramine	-8.17	1.02	-8.77
Etamiphylline	-6.55	15.75	-8.04
Moupinamide	-8.98	0.262	-11.36
N-cis-ferulotyramine	-8.02	1.32	-10.41
Petasiphinol	-8.70	0.422	-11.98

***FDA Approved Drug**

Anisotine is observed to have greater binding affinity(-9.56 kcal/mol) towards PI3K compared to the FDA approved drug alpelisib(-9.18 kcal/mol).

Anisotine forms one conventional hydrogen bond at Val882, seven van der Waals interactions, two pi-sigma bonds at Ile831 and Ile879. Four alkyl bonds are observed Trp812, Lys833, Leu838 and Ile963.

Two carbon-hydrogen bonds are observed at Asp836 and Glu880, with a pi-sulfur bond being formed at Met953.

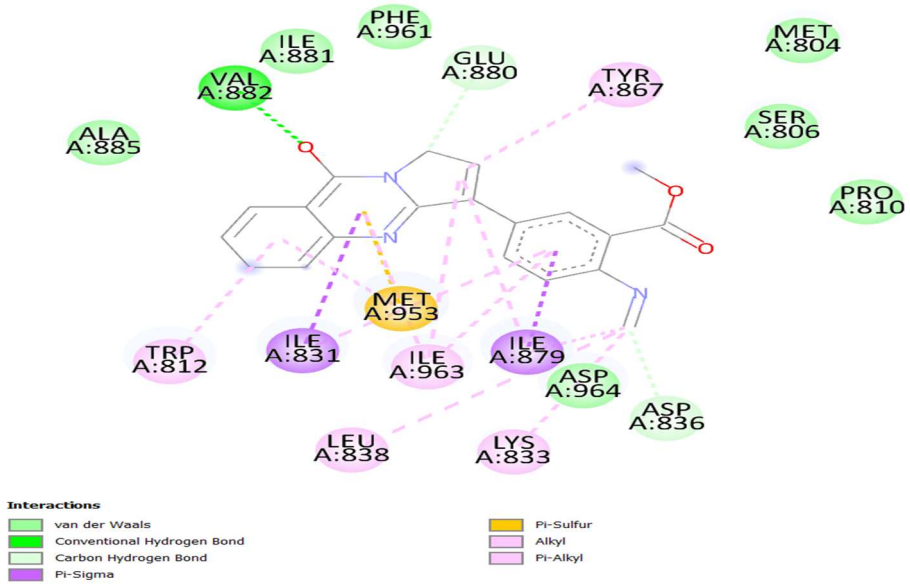


Fig 4.4: Interaction between anisotine and PI3K

4.2.4 DHFR

TABLE 4.4: Binding Energy of compounds against DHFR

Name of Compound	Binding Energy(kcal/mol)	Inhibition Constant(µmol)	Intermolecular Energy(kcal/mol)
Methotrexate*	-8.82	0.344	-12.69
Anisotine	-9.06	0.227	-10.26
Eseramine	-8.29	0.837	-8.89
Etamiphylline	-6.33	23.02	-7.82
Moupinamide	-7.74	2.11	-10.13
N-cis-ferulotyramine	-7.19	5.4	-9.57
Petasiphinol	-8.01	1.34	-11.29

***FDA Approved Drug**

Anisotine showed more binding affinity(-9.06 kcal/mol) than the FDA approved drug, methotrexate(-8.82 kcal/mol).

Anisotine forms six van der Waals interactions, two pi-pi T-shaped interactions at Phe31 and Phe34, two alkyl interactions at Leu22 and Leu67. Two carbon-hydrogen bonds are observed at Ile60 and Pro61.

Four conventional hydrogen bonds are observed at Ile7, Ala9, Val115 and Tyr121.

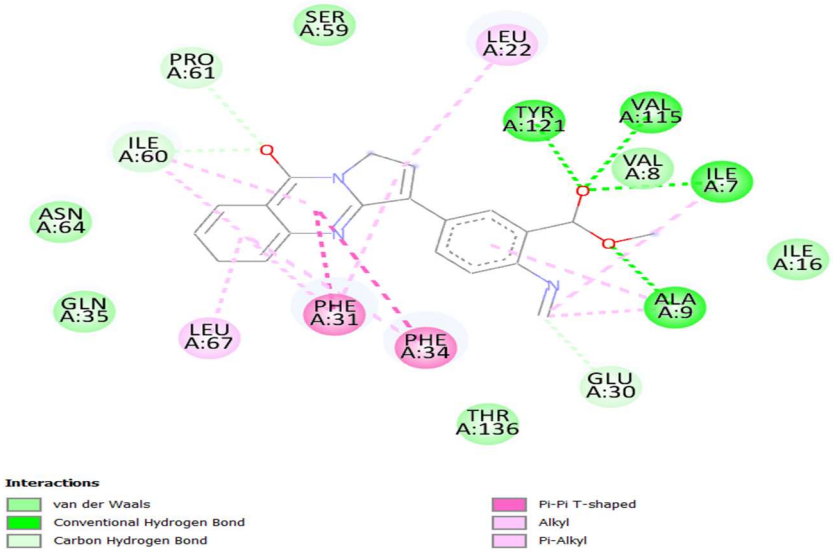


Fig 4.5: Interaction between anisotine and DHFR

4.2.5. NK-1R

TABLE 4.5: Binding Energy of compounds against NK-1R

Name of Compound	Binding Energy(kcal/mol)	Inhibition Constant(µmol)	Intermolecular Energy(kcal/mol)
Aprepitant*	-8.43	0.656	-10.82
Anisotine	-9.56	0.097	-10.76
Eseramine	-7.45	3.46	-8.05
Etamiphylline	-5.94	44.42	-7.43
Moupinamide	-7.90	1.61	-10.29
N-cis-ferulotyramine	-7.14	5.87	-9.52
Petasiphinol	-8.06	1.24	-11.34

***FDA Approved Drug**

Of all the compounds tested, anisotine(-9.56 kcal/mol) shows greater binding affinity for NK-1R than the FDA approved drug aprepitant(-8.43 kcal/mol).

Anisotine forms six van der Waals interactions with six amino acids, two alkyl bonds with Ile116 and Met295. It also forms two pi-sigma bonds with Val200 and Trp261.

In addition, three conventional hydrogen bonds at His197, Thr201 and His265 are observed, with pi-amide and pi stacked bonds with Pro112 and Phe264.

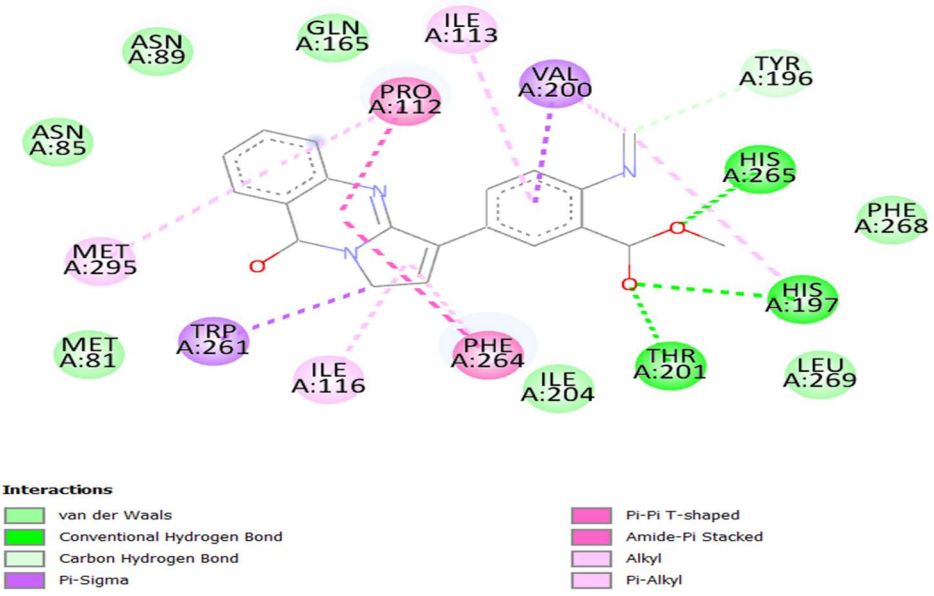


Fig 4.6: Interaction between Anisotine and NK-1R

The bioavailability and docking studies indicate that anisotine has a greater binding affinity for four receptors: aromatase, DHFR, CDK6 and NK-1R. The greater binding affinity indicates a more efficient binding between anisotine and the receptors compared to the FDA approved drugs used as reference, with an exception of CDK6, where it showed less affinity than the reference drug compound.

CHAPTER 5
DISCUSSION

Section 2.1 states the correlation between Hashimoto's thyroiditis and breast cancer development. Hypothyroidism caused by Hashimoto's thyroiditis, is known to reduce the concentrations of sex hormone binding globulin(SHBG). SHBG, binds to the free sex hormones, both estrogen and testosterone, to ensure their clearance from the body.

In conditions that reduce the concentrations of SHBG[26], it cannot bind efficiently to the sex hormones, and their clearance rate is reduced. Such a situation causes elevation in the levels of free sex hormones. E2 levels are known to correspond with prognosis of breast cancer development and increase of free estradiol aggravates the chance[25], with the development of ER positive breast cancer being observed in patients with Hashimoto's thyroiditis[22].

ER positive breast cancer metastasizes in the body using several receptors and targets, chief of which is aromatase, which can convert free testosterone to even more free E2, further increasing its levels. Aromatase inhibitors, such as anastrozole are able to halt the function of aromatase to limit estrogen synthesis, but are known to cause serious side effects which include development of autoimmune diseases such as SLE and vasculitis[16][34]. The patient being exposed to further autoimmune diseases in addition to Hashimoto's thyroiditis can result in development of multiple autoimmune syndrome(MAS)[79]. MAS is known to occur in about 25% of patients already suffering from an autoimmune disorder. Similar autoimmune disorder developments are seen in the administration of other FDA approved drugs for targets other than aromatase in breast cancer, such as abemaciclib[16], which targets CDK6.

In order to resolve this complication, newer drugs need to be discovered, with natural compounds being the best alternatives. Natural compounds are found in plants, several of which are being used in traditional medicine formulations for ages, without any serious side effects. Using the same principle, natural compounds can be discovered

which work on the target receptors as efficiently as the FDA approved drugs, without any risk of serious side effects.

The IMPPAT database provided a list of natural compounds, which were subjected to bioavailability testing, to generate lead compounds, which could be used as possible replacements and potential inhibitors.

Docking analysis provided the binding affinities for the phytochemicals and gave anisotine as the lead compound which bound four receptors: aromatase, DHFR, PI3K and NK-1R with greater affinity than the FDA approved drugs. Anisotine is obtained from *Justicia adhatoda*, also known as *Adhatoda zeylanica* and *Adhatoda vasica*, commonly called Malabar Nut, is used frequently in ayurvedic medicine, and clinical studies have been performed to determine the anticancer activity on lung cancer cell lines[80] and as an antioxidant[81]. Thus, it is proposed that anisotine can act as a potential inhibitor for multiple receptors for breast cancer, without manifestation of autoimmune disorders.

CHAPTER 6
CONCLUSION

The present study involved systematic curation of FDA approved drugs for breast cancer. The FDA approved drugs are currently used to treat breast cancer by targeting various pathways and receptors. Despite the numerous benefits of the FDA approved drugs, including providing a good disease-free survival time, numerous side effects exist, ranging from drug resistance to developing of further autoimmune diseases in breast cancer patients already suffering from Hashimoto's thyroiditis. Natural compounds are thus needed because synthetic drugs can cause side effects and since natural compounds are obtained from plants, and thus can be tolerated better by the body. Anisotine, which is isolated from *Justicia adhatoda*, satisfied all screening parameters and showed better binding affinity than FDA drugs for four different receptors, and can be a viable replacement for FDA approved synthetic drugs with same affinity towards the target receptors and lower chance of causing side-effects.

REFERENCES

[1] S. Rahman *et al.*, "Molecular insights into the relationship between autoimmune thyroid diseases and breast cancer: A critical perspective on autoimmunity and ER stress," *Front. Immunol.*, vol. 10, no. MAR, 2019, doi: 10.3389/fimmu.2019.00344.

[2] C. Giuliani, I. Bucci, and G. Napolitano, "The role of the transcription factor Nuclear Factor-kappa B in thyroid autoimmunity and cancer," *Front. Endocrinol. (Lausanne).*, vol. 9, no. AUG, pp. 1–8, 2018, doi: 10.3389/fendo.2018.00471.

[3] F. Ragusa *et al.*, "Hashimotos' thyroiditis: Epidemiology, pathogenesis, clinic and therapy," *Best Pract. Res. Clin. Endocrinol. Metab.*, vol. 33, no. 6, p. 101367, Dec. 2019, doi: 10.1016/j.beem.2019.101367.

[4] P. Caturegli, A. De Remigis, and N. R. Rose, "Hashimoto thyroiditis: Clinical and diagnostic criteria," *Autoimmun. Rev.*, vol. 13, no. 4–5, pp. 391–397, 2014, doi: 10.1016/j.autrev.2014.01.007.

[5] L. Chaker, A. C. Bianco, J. Jonklaas, and R. P. Peeters, "Hypothyroidism," *Lancet*, vol. 390, no. 10101, pp. 1550–1562, Sep. 2017, doi: 10.1016/S0140-6736(17)30703-1.

[6] B. Vaidya, Chakera, and Pearce, "Treatment for primary hypothyroidism: current approaches and future possibilities," *Drug Des. Devel. Ther.*, vol. 6, p. 1, Dec. 2011, doi: 10.2147/DDDT.S12894.

[7] H. B. Burch and D. S. Cooper, "Management of Graves Disease," *JAMA*, vol. 314, no. 23, p. 2544, Dec. 2015, doi: 10.1001/jama.2015.16535.

[8] R. Desailloud and D. Hober, "Viruses and thyroiditis: An update," *Virol. J.*, vol. 6, 2009, doi: 10.1186/1743-422X-6-5.

[9] T. P. Foley and M. Charron, “Radioiodine treatment of juvenile Graves disease,” *Exp. Clin. Endocrinol. Diabetes*, vol. 105, no. S 04, pp. 61–65, Jul. 2009, doi: 10.1055/s-0029-1211936.

[10] H. B. Burch, K. D. Burman, and D. S. Cooper, “A 2011 Survey of Clinical Practice Patterns in the Management of Graves’ Disease,” *J. Clin. Endocrinol. Metab.*, vol. 97, no. 12, pp. 4549–4558, Dec. 2012, doi: 10.1210/jc.2012-2802.

[11] Z. W. Liu, L. Masterson, B. Fish, P. Jani, and K. Chatterjee, “Thyroid surgery for Graves’ disease and Graves’ ophthalmopathy,” *Cochrane Database Syst. Rev.*, vol. 2015, no. 11, 2015, doi: 10.1002/14651858.CD010576.pub2.

[12] Y. S. Sun *et al.*, “Risk factors and preventions of breast cancer,” *Int. J. Biol. Sci.*, vol. 13, no. 11, pp. 1387–1397, 2017, doi: 10.7150/ijbs.21635.

[13] W. Majeed *et al.*, “MINI-REVIEW Breast Cancer : Major Risk Factors and Recent Developments in Treatment,” *Asian Pac J Cancer Prev*, vol. 15, pp. 3353–3358, 2014, doi: 10.7314/APJCP.2014.15.8.3353.

[14] T. Bremec, J. Demsar, B. Luzar, and M. D. Pavlović, “Drug-induced pruritic micropapular eruption: anastrozole, a commonly used aromatase inhibitor.,” *Dermatol Online J*, vol. 15, no. 7, 2009.

[15] O. Klapko, E. Ghoulam, S. Jakate, S. Eswaran, and L. Usha, “Anastrozole-induced autoimmune hepatitis: A rare complication of breast cancer therapy,” *Anticancer Res.*, vol. 37, no. 8, pp. 4173–4176, 2017, doi: 10.21873/anticanres.11805.

[16] D. J. Kurtyka, A. D. Mohebbi, K. T. Burke, and M. A. Cardis, “Subacute cutaneous lupus erythematosus following abemaciclib therapy for metastatic breast cancer,” *JAAD Case Reports*, vol. 14, pp. 10–12, 2021, doi: 10.1016/j.jdcr.2021.05.028.

[17] M. Trancart, A. Cavailhes, B. Balme, and F. Skowron, “Anastrozole-induced subacute cutaneous lupus erythematosus,” *Br J Dermatol*, vol. 158, no. 3, pp. 628–629, 2008, doi: 10.1111/j.1365-2133.2007.08367.x.

[18] T. Ozmen, B. M. Gulluoglu, C. S. Yegen, and A. Soran, “Autoimmune Thyroid Disease and Breast Cancer Prognosis,” *J. Breast Heal.*, vol. 11, no. 2,

pp. 67–71, 2015, doi: 10.5152/tjbh.2015.2462.

[19] I. Muller *et al.*, “High prevalence of breast cancer in patients with benign thyroid diseases,” *J. Endocrinol. Invest.*, vol. 34, no. 5, pp. 349–352, 2011, doi: 10.3275/7141.

[20] G. Graceffa *et al.*, “Breast cancer in previously thyroidectomized patients: Which thyroid disorders are a risk factor?,” *Futur. Sci. OA*, vol. 7, no. 5, 2021, doi: 10.2144/fsoa-2021-0029.

[21] L. Bach, K. Kostev, L. Schiffmann, and M. Kalder, “Association between thyroid gland diseases and breast cancer: a case–control study,” *Breast Cancer Res. Treat.*, vol. 182, no. 1, pp. 207–213, 2020, doi: 10.1007/s10549-020-05675-6.

[22] C. Chiappa *et al.*, “Breast cancer and thyroid diseases: analysis of 867 consecutive cases,” *J. Endocrinol. Invest.*, vol. 40, no. 2, pp. 179–184, 2017, doi: 10.1007/s40618-016-0543-4.

[23] I. Mohammad *et al.*, “Estrogen receptor α contributes to T cell–mediated autoimmune inflammation by promoting T cell activation and proliferation,” *Sci. Signal.*, vol. 11, no. 526, Apr. 2018, doi: 10.1126/scisignal.aap9415.

[24] R. Hampl, R. Kancheva, M. Hill, M. Bičíková, and K. Vondra, “Interpretation of Sex Hormone-Binding Globulin Levels in Thyroid Disorders,” *Thyroid*, vol. 13, no. 8, pp. 755–760, Aug. 2003, doi: 10.1089/105072503768499644.

[25] . Endogenous Hormones and Breast Cancer Collaborative Group, “Sex hormones and risk of breast cancer in premenopausal women: a collaborative reanalysis of individual participant data from seven prospective studies,” *Lancet Oncol.*, vol. 14, no. 10, pp. 1009–1019, Sep. 2013, doi: 10.1016/S1470-2045(13)70301-2.

[26] S. S. Tworoger, S. A. Missmer, R. L. Barbieri, W. C. Willett, G. A. Colditz, and S. E. Hankinson, “Plasma Sex Hormone Concentrations and Subsequent Risk of Breast Cancer Among Women Using Postmenopausal Hormones,” *Cancer Epidemiol Biomarkers Prev* ., vol. 12, no. 12, pp. 1457–1461, 2003.

[27] S. Noble, R. Simpson, and E. Bulun, “Aromatase expression in

endometriosis," *J Clin Endocrinol Metab*, vol. 450, pp. 174–179, 1996.

[28] K. Toda and Y. Shizuta, "Molecular cloning of a cDNA showing alternative splicing of the 5′-untranslated sequence of mRNA for human aromatase P-450 Enhanced Reader.pdf," *Eur. J. Biochem*, vol. 213, pp. 383–389, 1993, doi: 10.1111/j.1432-1033.1993.tb17772.x.

[29] H. Zhao, L. Zhou, A. J. Shangguan, and S. E. Bulun, "Aromatase expression and regulation in breast and endometrial cancer," *J. Mol. Endocrinol.*, vol. 57, no. 1, pp. R19–R33, 2016, doi: 10.1530/JME-15-0310.

[30] D. Ghosh, S. Hubbell, C. Egbuta, W. Jiang, J. An, and M. L. Huw, "Novel Aromatase Inhibitors by Structure-Guided Design," *J Med Chem*, vol. 55, no. 19, pp. 8464–8476, 2013, doi: 10.1021/jm300930n.Novel.

[31] S. E. Bulun *et al.*, "Regulation of Aromatase Expression in Estrogen-Responsive Breast and Uterine Disease : From Bench to Treatment," *Pharmacol. Rev.*, vol. 57, no. 3, pp. 359–383, 2005, doi: 10.1124/pr.57.3.6.359.

[32] B. E. Henderson and H. S. Feigelson, "Hormonal carcinogenesis," *Carcinogenesis*, vol. 21, no. 3, pp. 427–433, 2000.

[33] J. Cuzick *et al.*, "Use of anastrozole for breast cancer prevention (IBIS-II): long-term results of a randomised controlled trial," *Lancet*, vol. 395, no. 10218, pp. 117–122, 2020, doi: 10.1016/S0140-6736(19)32955-1.

[34] M. Wong, J. Grossman, B. H. Hahn, and A. La Cava, "Cutaneous vasculitis in breast cancer treated with chemotherapy," pp. 3–9, 2008, doi: 10.1016/j.clim.2008.07.001.

[35] A. Deshpande, P. Sicinski, and P. W. Hinds, "Cyclins and cdks in development and cancer: A perspective," *Oncogene*, vol. 24, no. 17, pp. 2909–2915, 2005, doi: 10.1038/sj.onc.1208618.

[36] È. Parasorbosid *et al.*, "Crystal structure of the complex of the cyclin D-dependent kinase Cdk6 bound to the cell-cycle," *Nature*, vol. 396, no. November, pp. 13–20, 1998.

[37] Y. S. Cho *et al.*, "Fragment-Based Discovery of 7-Azabenzimidazoles as

Potent, Highly Selective, and Orally Active CDK4/6 Inhibitors," vol. 100, 2012.

[38] F. Mohammadizadeh, M. Hani, M. Ranaee, and M. Bagheri, "Role of cyclin D1 in breast carcinoma," *J Res Med Sci*, no. December, pp. 1021–1025, 2013.

[39] M. Piezzo *et al.*, "Targeting cell cycle in breast cancer: CDK4/6 inhibitors," *Int. J. Mol. Sci.*, vol. 21, no. 18, pp. 1–23, 2020, doi: 10.3390/ijms21186479.

[40] K. Pandey *et al.*, "Molecular mechanisms of resistance to CDK4/6 inhibitors in breast cancer: A review," *Int. J. Cancer*, vol. 145, no. 5, pp. 1179–1188, 2019, doi: 10.1002/ijc.32020.

[41] C. Yang *et al.*, "Acquired CDK6 amplification promotes breast cancer resistance to CDK4 / 6 inhibitors and loss of ER signaling and dependence," *Oncogene*, vol. 36, no. June 2016, pp. 2255–2264, 2017, doi: 10.1038/onc.2016.379.

[42] C. L. Braal, E. M. Jongbloed, S. M. Wilting, R. H. J. Mathijssen, S. L. W. Koolen, and A. Jager, "Inhibiting CDK4/6 in Breast Cancer with Palbociclib, Ribociclib, and Abemaciclib: Similarities and Differences," *Drugs*, vol. 81, no. 3, pp. 317–331, 2021, doi: 10.1007/s40265-020-01461-2.

[43] D. A. Matthews *et al.*, "Dihydrofolate reductase: X-ray structure of the binary complex with methotrexate," *Science (80-.).*, vol. 197, no. 4302, pp. 452–455, 1977, doi: 10.1126/science.17920.

[44] M. R. Sawaya and J. Kraut, "Loop and subdomain movements in the mechanism of Escherichia coli dihydrofolate reductase: Crystallographic evidence," *Biochemistry*, vol. 36, no. 3, pp. 586–603, 1997, doi: 10.1021/bi962337c.

[45] V. Cody, J. R. Luft, A. Gangjee, and S. F. Queener, "Structure determination of tetrahydroquinazoline antifolates in complex with human and Pneumocystis carinii dihydrofolate reductase : correlations between enzyme selectivity and stereochemistry research papers," *Acta Crystallogr. Sect. D*, pp. 646–655, 2004, doi: 10.1107/S0907444904002094.

[46] R. M. Levine, E. Rubalcaba, M. E. Lippman, and K. H. Cowan, "Effects of

estrogen and tamoxifen on the regulation of dihydrofolate reductase gene expression in a human breast cancer cell line," 1985.

[47] M. Nakano, T. Fukami, S. Gotoh, and M. Nakajima, "A-to-I RNA editing up-regulates human dihydrofolate reductase in breast cancer," *J. Biol. Chem.*, vol. 292, no. 12, pp. 4873–4884, 2017, doi: 10.1074/jbc.M117.775684.

[48] V. Yang *et al.*, "Breast cancer: Insights in disease and influence of drug methotrexate," *RSC Med. Chem.*, vol. 11, no. 6, pp. 646–664, 2020, doi: 10.1039/d0md00051e.

[49] Y. S. Ong, M. Bañobre-López, S. A. Costa Lima, and S. Reis, "A multifunctional nanomedicine platform for co-delivery of methotrexate and mild hyperthermia towards breast cancer therapy," *Mater. Sci. Eng. C*, vol. 116, p. 111255, Nov. 2020, doi: 10.1016/j.msec.2020.111255.

[50] S. van Outryve *et al.*, "Methotrexate-associated liver toxicity in a patient with breast cancer: case report and literature review," *Neth J Med*, vol. 60, no. 5, pp. 216–222, 2002.

[51] P. Koźmiński, P. K. Halik, R. Chesori, and E. Gniazdowska, "Overview of dual-acting drug methotrexate in different neurological diseases, autoimmune pathologies and cancers," *Int. J. Mol. Sci.*, vol. 21, no. 10, 2020, doi: 10.3390/ijms21103483.

[52] D. Miricescu, A. Totan, I. I. Stanescu-Spinu, S. C. Badoiu, C. Stefani, and M. Greabu, "PI3K/AKT/mTOR signaling pathway in breast cancer: From molecular landscape to clinical aspects," *Int. J. Mol. Sci.*, vol. 22, no. 1, pp. 1–24, 2021, doi: 10.3390/ijms22010173.

[53] K. Araki and Y. Miyoshi, "Mechanism of resistance to endocrine therapy in breast cancer: the important role of PI3K/Akt/mTOR in estrogen receptor-positive, HER2-negative breast cancer," *Breast Cancer*, vol. 25, no. 4, pp. 392–401, 2018, doi: 10.1007/s12282-017-0812-x.

[54] N. Vasan, E. Toska, and M. Scaltriti, "Overview of the relevance of PI3K pathway in HR-positive breast cancer," *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.*, vol. 30, no. 10, pp. x3–x11, 2019, doi: 10.1093/annonc/mdz281.

[55] D. P. Sutherlin *et al.*, “Discovery of a Potent , Selective , and Orally Available Class I Phosphatidylinositol 3-Kinase (PI3K)/ Mammalian Target of Rapamycin (mTOR) Kinase Inhibitor (GDC-0980) for the Treatment of Cancer,” *J. Med. Chem.*, pp. 7579–7587, 2011.

[56] S. E. Nunnery and I. A. Mayer, “Targeting the PI3K/AKT/mTOR Pathway in Hormone-Positive Breast Cancer,” *Drugs*, vol. 80, no. 16, pp. 1685–1697, 2020, doi: 10.1007/s40265-020-01394-w.

[57] I. A. Mayer *et al.*, “A Phase Ib Study of Alpelisib (BYL719), a PI3Ka-Specific Inhibitor, with Letrozole in ERþ/HER2 Metastatic Breast Cancer,” *Clin. Cancer Res.*, vol. 23, no. 1, pp. 26–34, Jan. 2017, doi: 10.1158/1078-0432.CCR-16-0134.

[58] H. Ellis and C. X. Ma, “PI3K Inhibitors in Breast Cancer Therapy,” *Curr. Oncol. Rep.*, vol. 21, no. 12, 2019, doi: 10.1007/s11912-019-0846-7.

[59] A. B. Hanker, V. Kaklamani, and C. L. Arteaga, “Challenges for the Clinical Development of PI3K Inhibitors: Strategies to Improve Their Impact in Solid Tumors,” *Cancer Discov.*, vol. 9, no. 4, pp. 482–491, Apr. 2019, doi: 10.1158/2159-8290.CD-18-1175.

[60] C. A. Maggi, “The Mammalian Tachykinin Receptors,” *Gen. Pharmac*, vol. 26, no. 5, pp. 911–944, 1995, doi: 10.1016/0306-3623(94)00292-U.

[61] J. Yin *et al.*, “Crystal structure of the human NK 1 tachykinin receptor,” 2018, doi: 10.1073/pnas.1812717115.

[62] M. S. Steinhoff, B. Von Mentzer, P. Geppetti, C. Pothoulakis, and N. W. Bunnett, “TACHYKININS AND THEIR RECEPTORS : AND THE MECHANISMS OF DISEASE,” no. 341, pp. 265–301, 2022, doi: 10.1152/physrev.00031.2013.

[63] S. J. Baker, J. L. Morris, and I. L. Gibbins, “C loning of a C-terminally truncated NK-1 receptor from guinea-pig nervous system q,” vol. 111, pp. 136–147, 2003.

[64] C. Thom *et al.*, “Structures of neurokinin 1 receptor in complex with G q and G s proteins reveal substance P binding mode and unique activation features,”

pp. 30–32, 2021.

[65] M. Rosso, M. Mu, and M. Berger, “The Role of Neurokinin-1 Receptor in the Microenvironment of Inflammation and Cancer,” *Sci. World J.*, vol. 2012, 2012, doi: 10.1100/2012/381434.

[66] I. M. Hennig and J. A. Laissue, “Substance-p receptors in human primary neoplasms: tumoral and vascular localization,” *Int J. Cancer*, vol. 792, pp. 786–792, 1995, doi: 10.1002/ijc.2910610608.

[67] F. Entschladen, Æ. K. Lang, Æ. T. L. Drell, J. Joseph, and Æ. K. S. Zaenker, “Neurotransmitters are regulators for the migration of tumor cells and leukocytes,” *Cancer Immunol Immunother*, vol. 51, pp. 467–482, 2002, doi: 10.1007/s00262-002-0300-8.

[68] A. He, J. M. Alhariri, R. J. Sweren, M. M. Kwatra, and S. G. Kwatra, “Aprepitant for the Treatment of Chronic Refractory Pruritus,” *Biomed Res. Int.*, vol. 2017, pp. 1–6, 2017, doi: 10.1155/2017/4790810.

[69] L. V. dos Santos, F. H. Souza, A. T. Brunetto, A. D. Sasse, and J. P. da Silveira Nogueira Lima, “Neurokinin-1 Receptor Antagonists for Chemotherapy-Induced Nausea and Vomiting: A Systematic Review,” *JNCI J. Natl. Cancer Inst.*, vol. 104, no. 17, pp. 1280–1292, Sep. 2012, doi: 10.1093/jnci/djs335.

[70] M. Mayuri and T. M. Vijayakumar, “A comprehensive review on neurokinin-1 receptor antagonism in breast cancer,” vol. 11, no. 05, pp. 9–14, 2021, doi: 10.7324/JAPS.2021.110502.

[71] I. Beirith *et al.*, “Identification of the Neurokinin-1 Receptor as Targetable Stratification Factor for Drug Repurposing in Pancreatic Cancer,” 2021.

[72] D. Schneidman-Duhovny, O. Dror, Y. Inbar, R. Nussinov, and H. J. Wolfson, “PharmaGist: a webserver for ligand-based pharmacophore detection,” *Nucleic Acids Res.*, vol. 36, no. Web Server, pp. W223–W228, May 2008, doi: 10.1093/nar/gkn187.

[73] D. R. Koes and C. J. Camacho, “ZINCPharmer: pharmacophore search of the ZINC database,” *Nucleic Acids Res.*, vol. 40, no. W1, pp. W409–W414, Jul.

2012, doi: 10.1093/nar/gks378.

[74] J. J. Irwin and B. K. Shoichet, “ZINC – A Free Database of Commercially Available Compounds for Virtual Screening,” vol. 45, no. 1, pp. 177–182, 2006.

[75] K. Mohanraj *et al.*, “IMPPAT: A curated database of Indian Medicinal Plants, Phytochemistry And Therapeutics,” *Sci. Rep.*, vol. 8, no. 1, p. 4329, Dec. 2018, doi: 10.1038/s41598-018-22631-z.

[76] S. K. Burley *et al.*, “RCSB Protein Data Bank: powerful new tools for exploring 3D structures of biological macromolecules for basic and applied research and education in fundamental biology, biomedicine, biotechnology, bioengineering and energy sciences,” *Nucleic Acids Res.*, vol. 49, no. D1, pp. D437–D451, Jan. 2021, doi: 10.1093/nar/gkaa1038.

[77] A. Daina, O. Michielin, and V. Zoete, “SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules,” *Sci. Rep.*, vol. 7, no. 1, p. 42717, May 2017, doi: 10.1038/srep42717.

[78] C. A. Lipinski, “Lead- and drug-like compounds: the rule-of-five revolution,” *Drug Discov. Today Technol.*, vol. 1, no. 4, pp. 337–341, Dec. 2004, doi: 10.1016/j.ddtec.2004.11.007.

[79] M. Cojocaru, M. I. Cojocaru, and I. Silosi, “Multiple autoimmune disorder,” *Maedica (Buchar.)*, vol. 5, no. 2, pp. 132–134, 2010.

[80] D. Latha *et al.*, “Facile Justicia adhatoda leaf extract derived route to silver nanoparticle: synthesis, characterization and its application in photocatalytic and anticancer activity,” *Mater. Res. Express*, vol. 6, 2019.

[81] R. P. Singh, B. Padmavathi, and A. R. Rao, “Modulatory influence of Adhatoda vesica(Justicia adhatoda) leaf extract on the enzymes of xenobiotic metabolism , antioxidant status and lipid peroxidation in mice,” *Mol. Cell. Biochem.*, vol. 213, pp. 99–109, 2000.

APPENDICES

Appendix A: Supplementary Information

TABLE S1: Bioavailability parameters of all screened compounds from IMPPAT Database

Molecule	MW	Lipinski violations	Bioavailability Score	Lead violations	Synthetic Accessibility
4-Aminospiroperidol	410.48	0	0.55	1	3.9
N-(p-Aminophenethyl) piperone	514.63	1	0.55	3	4.61
AC1N79AY	379.41	0	0.55	2	3.01
Bottromycin A2	823.06	2	0.17	3	8.47
3-Hydroxy-3-methylglutaryl Coenzyme A	893.64	3	0.11	2	7.1
aminophylline	420.43	1	0.55	1	2.99
Isobutyryl CoA	837.62	3	0.11	2	6.81
AC1LA6SK	750	1	0.55	3	6.66
Etamiphylline	279.34	0	0.55	0	2.73
Ketanserin	395.43	0	0.55	1	3.25
NPPB	300.31	0	0.56	1	2.28
Nitrofurantoin	238.16	0	0.55	1	3.1

Cefixime	453.45	1	0.11	2	4.81
Cefepime	480.56	1	0.55	2	5.09
AC1N75QA	348.82	1	0.55	1	3.73
Cosmosin	513.59	2	0.11	2	4.9
Nitroblue tetrazolium chloride	817.64	3	0.17	3	4.89
Thyrotropin releasing hormone	362.38	0	0.55	2	3.76
Eseramine	318.37	0	0.55	0	3.63
AC1N1DX2	402.89	0	0.55	2	3.65
Bay K 8644	356.3	0	0.55	1	4.03
Cyclosulfamuron	421.43	1	0.55	2	3.32
Aspartyl-proline	230.22	0	0.56	1	2.62
Anisotine	349.38	0	0.55	0	3.38
AC1MHGUQ	310.86	0	0.55	1	2.17
Imatinib	493.6	0	0.55	3	3.78
Thifensulfuron	373.36	1	0.11	1	3.45
N-ferutolylserotonin	352.38	0	0.55	1	2.72
1,2-Dihydrocurcumin	370.4	0	0.55	2	3.1
Dicaffeoylputrescene	412.44	1	0.55	2	2.94
Cimiracemate B	358.34	0	0.55	2	2.92
Terrestribisamide	440.49	0	0.55	2	3.18
Petasiphinol	344.32	0	0.55	0	2.82
n-cis-feruloyltyramine	313.35	0	0.55	0	2.55
Moupinamide	313.35	0	0.55	0	2.55

APPENDIX B: LIST OF PUBLICATIONS

- Dhingra M*., Mahalanobis S.* and Das A. Thyroid Receptor β might be responsible for breast cancer associated with Hashimoto's Thyroiditis: A new insight into pathogenesis. Accepted in Immunologic Research(Springer). Acceptance Date: April 19, 2022

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We are pleased to inform you that your submission Thyroid Receptor β is responsible for breast cancer associated with Hashimoto's thyroiditis: A new insight. has been accepted for publication in Immunologic Research

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- Dhingra M., Mahalanobis S. and Das A. Isowogonin obtained from *Didymocarpus pedicellata* prevents cholinergic syndrome associated with Irinotecan in treatment of pancreatic cancer. Submitted in International Conference on Chemical, Agricultural, Biological and Environmental Sciences(24 April), New Delhi.

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21st - 22nd April, 2022 at New Delhi, India

EVENT ACCEPTANCE LETTER

Dear Muskaan Dhingra,Shayon Mahalanobis and Asmita Das

We are happy to inform you that your PAPER has been selected for ICCABES on 21st - 22nd April, 2022 at New Delhi, India after peer review process which will be organized by **GSRD**and in association with **PET** for presentation (oral presentation/ poster presentation) at the Conference. Registered paper/Abstract will get Conference Proceeding having ISBN *(International Standard Book Number)* and certificates of presentation.

Paper Title: *Isowogonin obtained from Didymocarpus pedicellataprevents cholinergic syndrome associated with Irinotecan in treatment of pancreatic cancer*

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- Mahalanobis S., Apoorva, Sharma N., Luthra R., Menon T., and Das A., "Cancer Prognosis and Immune System," in *Microbial Crosstalk with Immune System*. To be published by Elsevier.

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2K20/MSCBIO/29

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I hereby certify that the dissertation project titled "**Elucidation of natural compounds interfering with Hashimoto's thyroiditis mediated induction of breast cancer**" which is submitted by **Shayon Mahalanobis, 2K20/MSCBIO/29**, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of requirement for the award of the degree of Master of Science, is a record of work carried out by the student under my supervision. To the best of my knowledge, this work has not been submitted in part of any Degree or Diploma to this University or elsewhere.

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