"In-vitro Evaluation and Characterization of *Avicennia marina* Whole Leaf Extract along with the In-silico Approach for Identification of Effective Inhibitors for HER2 Positive Breast Cancer from *A. marina* Leaf Phytochemicals"

A THESIS

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CERTIFICATE OF RECOMMENDATION

We hereby recommend that the thesis entitled ***"In-vitro Evaluation and Characterization of Avicennia marina Whole Leaf Extract along with the In-silico Approach for Identification of Effective Inhibitors for HER2 Positive Breast Cancer from A. marina Leaf Phytochemicals"*** carried out under my supervision by Ananya Das may be accepted in partial fulfilment of the requirement for awarding the Degree of Master in Biomedical Engineering of Jadavpur University. The project, in our opinion, is worthy for its acceptance.

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The forgoing thesis is hereby approved as a creditable study of an engineering subject carried out and presented in a manner satisfactory to warrant its acceptance as a prerequisite to the degree for which it has been submitted. It is understood that by this approval the undersigned do not necessarily endorse or approve any statement made, opinion expressed or conclusion drawn therein but approve the thesis only for the purpose for which it is submitted.

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of his **Master of Engineering in Biomedical Engineering** studies during academic session 2020-2022.

All information in this document has been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that, as required by this rules and conduct, I have fully cited and referred all material and results that are not original to this work.

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Date: ***Ananya Das***

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Chapter 1

Abstract

Abstract:

Cancer being one of the deadliest diseases in the world has always been an important topic for medical researches. Still there's no hundred percent safe anti-cancer therapy, for treatment of cancer. Chemotherapy, the most popular anti-cancer therapy always has toxic and adverse side effects. Therefore natural product based anti-cancer treatment which is a much safer way of cancer treatment has been started to get explored. Many plant extracts have shown significant level of anti-cancer potency without any side effects. *Avicennia marina* being an ancient and major mangrove plant has been used in treating different diseases for a long time. Several studies have shown that extract of this plant has anticancer effect against many forms of cancer manifested in human body like lung cancer, colon cancer, liver cancer etc. [1] [2] Breast cancer is one of the very common types of cancer in human females. There is less sufficient data about the anti-cancer potential of *A. marina* plant extract against breast cancer. So, the present study was done based on *Avicennia marina* whole leaf extract to evaluate the anticancer properties of it against breast cancer (in vitro characterization of leaf and in silico leaf phytochemical analysis along with their docking with HER2+ breast cancer specific protein HER2).

The present study was conducted by phytochemical screening, estimation of total phenol and flavonoid content, antibacterial assay, antioxidant or free radical scavenging assay (RSA) using DPPH, anti-inflammatory assay using Bovine Serum Albumin (BSA), cytotoxic study (MTT assay) against normal fibroblast cell line (L929) and hemocompatibility test. The total phenol and flavonoid content revealed the phytochemical potency of the whole leaf extract of A. marina. The RSA% of A. marina leaf extract against free radical (DPPH) was compared with standard Ascorbic acid. MTT assay result showed that A. marina leaf extract promoted the growth of non-tumor, healthy cells (L929). The anticancer property of A. marina whole leaf extract may be due to apoptosis of the cancerous cells, initiated by its phytochemical content (i.e. phenols, flavonoids) and its antioxidant properties. Therefore in silico study was done based on A. marina leaf specific phytochemicals; their ADMET properties were screened and then selected ligands were docked against a breast cancer protein, HER2. HER2 has been chosen as the receptor because very few studies have been conducted on this particular breast cancer protein following the in silico approach to find out potential anti-cancer drug candidates.

Keywords: *Avicennia marina*, leaf, breast cancer, Phytochemicals, anti-oxidant, MTT, In-silico, docking, HER2

References:

1. Albinhassan, Tahani & Saleh, KamelA & Barhoumi, Zouhaier & Alshehri, MohammedAli & Al-Ghazzawi, AdelM. (2021). Anticancer, anti-proliferative activity of Avicennia marina plant extracts. Journal of Cancer Research and Therapeutics. 17. 879. 10.4103/jcrt.JCRT_659_19.

2. Huang, C., Lu, C. K., Tu, M. C., Chang, J. H., Chen, Y. J., Tu, Y. H., & Huang, H. C. (2016). Polyphenol-rich Avicennia marina leaf extracts induce apoptosis in human breast and liver cancer cells and in a nude mouse xenograft model. *Oncotarget*, *7*(24), 35874–35893. https://doi.org/10.18632/oncotarget.8624

Chapter 2

Introduction and Literature Review

2.1. Introduction:

2.1.1. Pharmacology: The science of drugs is known as **'Pharmacology'** which has been originated from two Greek words: 'Pharmacon' means 'drug' & 'logos' means 'discourse in'. This domain of study deals with the interactions of exogenously administered chemicals with the living systems. Whichever chemical molecule interacts with a living system and produces a biological response, can be considered as a drug. Pharmacology deals with all the aspects of these drugs including their safe and effective use for medical purposes. [1]

'Drug' this word is derived from French word 'Drogue' meaning 'a dry herb'. It is considered as a single active chemical compound that stays within a medicine prescribed for diagnosing, preventing, and treating a specific disease. Based on the definition of drug as described by WHO (1966) it is understood that a drug can literally be any compound that has its use for modification and exploration of different physiological systems or pathological states for the patient's wellbeing. [1]

Pharmacology consists of two main divisions- a) Pharmacodynamics and b) Pharmacokinetics.

a) ***Pharmacodynamics (PD):*** This word has originated from two Greek words- 'Pharmacon'-drug & 'dynamis'-power. This branch of study deals with-'what is done by the drug to our body'. The bio-chemical, physiological effects of drug on the body, the mechanism of action of drugs on organ system, cellular-, sub cellular- and macromolecular- levels; all these aspects of studies are included here. PD explores and explains the relationship between concentration of drug at the action site and the resulting effect, including time duration and intensity of therapeutic agent and adverse effects of the drug on the living system. A drug's binding with a receptor, determines the effect of that drug present at the action site. [1]

b) ***Pharmacokinetics (PK):*** This word has been derived from two Greek words- 'Pharmacon'-drug & 'Kinesis'- movement. This branch deals with- 'what the body does to the drug'. PK can be thought as a drug's journey through the living system or body. During the journey, a drug has to pass through four phases. These four properties of a drug are known as ADME property. They are as follows-

i) Absorption- deals with how the drug moves from its site of administration to the site of action,

ii) Distribution- deals with how the drug travels to different organs through the blood circulation system of our body.

iii) Metabolism- the step where drug is broken down by different biochemical processes.

iv) Excretion- this step describes the elimination of the drug from our body.

These properties are extensively studied while a new drug is being discovered for future clinical use. [2]

2.1.2. Drug Discovery: Introduction of a new drug to the market is very time-consuming and complex process and costs a huge amount of money for the pharmaceutical companies. In general, around ten years of research is needed to develop a clinically administrable drug. Drug discovery process generally involves certain defined stages. Those stages are as follows-

i. Target Discovery: This is the first phase of drug discovery process. In general, with the help of in vitro research, targets which are involved in causing specific diseases are first identified. A target is usually a molecule involved as an integral part of gene regulation and intracellular signalling. Target can be a nucleic acid sequence or a protein molecule. But the foremost criterion of a molecule to be regarded as a target is that it should be a "druggable" molecule which means, its activity can be manipulated by an exogenous compound (i.e. the drug). [3]

ii. Target Validation: After the potential target selection is done, it is needed for the researchers to show or prove that the target is most definitely involved in the progression of the disease under study and its activity can be regulated. Precise target validation experiments are conducted for the success of drug development in the later stages. Main goals of this stage are to validate the primary hypothesis through gene knockdowns, antibody interaction tests and to modulate the drug molecule's affinity towards target molecule by changing the molecular structure. [3]

iii. Lead Compound Identification: This is the third step in drug discovery process. The main work needs to be done in this stage is to identify and create a compound (i.e. the lead) which can interact with the target selected in the previous stage. Screening experiments can be conducted to identify naturally- occurring compounds which can be repurposed as drugs in future. Researchers can also design synthetic compounds as leads in the labs. But one thing should always be noted that this lead

compound must not interfere with other biological processes except the intended or desired ones. With the help of cell culture, not only mechanisms of the drug's action, but also certain initial safety tests are conducted at this stage. Pharmacokinetics and Pharmacodynamics of the particular drug are also precisely studied here. [3]

iv. Lead Optimization: After the lead compound (or lead compounds) is identified, it is important to get them optimized for better efficacy and safety purposes. The synthetic lead compounds can be designed in such a way that they should not interact with molecules other than the target. In this way off-target-binding of the leads can be prevented. Tests for optimisation of dose of the drug and best route for drug administration (oral/injection) are also done at this stage with the help of two dimensional or three dimensional cell culture platforms. Conduction of safety testing before introducing the drug in vivo model animals (like mice, rats etc.) are also done at this stage. But firstly all these tests should be done in 3D in vitro settings and then at the later stages, animal models are used. Thus ensuring better chances to develop a safe and effective drug. [3]

v. Preclinical Drug Development: This stage involves extensive tests on different animal models to monitor the side effects and if any side effect is observed, it is addressed accordingly. Animal testing is necessary before human trials to see whether the drug is safe for the human trials and whether the drug is working as expected or not. To go to the clinical trial stage, extensive tests and relevant dada are needed by the FDA (Food and Drug Administration). Animal models mimicking human system, e.g. knockouts or genetically-modified mice, are used for tests during this stage. [3]

vi. Investigational New Drug (IND) Application: Before going for clinical trials, an IND application must be submitted to the FDA. This document includes the following information-

- Toxicity and animal study related data
- Drug manufacturing information
- Data from previous human research related to this one
- Information about principle investigators who are conducting the whole drug discovery process.

After 30 days, going through the review procedure of the IND, FDA can respond in two ways-

- The IND gets approved and FDA consents to the clinical trials of the drug
- Or, FDA can put a temporary hold on clinical trials till further information is obtained which they had asked for, and sometimes they can even totally stop the whole process.

As up till now, a whole lot of money has been invested and intensive researches have been conducted, in general it is rare for the FDA to cancel an already submitted IND. They generally ask for further improvements or more researches before granting permission for clinical trials if the submitted IND doesn't fulfil all the necessary criteria. [3]

vii. Clinical Trials: There are three phases in clinical trials. Those are described briefly as follows-

1. Phase I Clinical Trials: At this phase the new drug is tested on 100 or lesser number of healthy human beings. This is done in order to find out the relative safety of the medicine. Carcinogenicity testing is done on animal models (Tg rasH2 mouse) to determine carcinogenic potential of the drug. Human c-Ha-ras oncogene and endogenous mouse Ha-ras oncogene both are present in the mouse model. As human copy of the gene is present in the mouse, the mouse model develops tumour after being exposed to carcinogenic compounds which causes cancer in human beings. Use of this special mouse model has reduced the carcinogenicity testing time to six months; previously it took two years at least to complete the testing.
2. Phase II Clinical Trials: Here the number of human patients gets increased to 100-500. These patients must be suffering from the disease that the newly develop drug is attempting to cure. Side effects, adverse effects and efficacy of the drug; all of these are tested in this phase.
3. Phase III Clinical Trials: Now the number of patients gets increased to a group of 1000-5000. This much number of patients is needed for generating a statistically significant data. Here the overall efficacy and safety is determined. Only 12% of the drugs had made through this phase as per report till today. [3]

viii. FDA review and Approval: After clinical trials are done, a NDA is submitted to the FDA. This document holds all the information about the new drug. Safety, efficacy, new dug's interactions with other medications, all clinical results, information about all the studies are included in this document. FDA takes 6 to 10 months' time for reviewing

the NDA. If the drug is approved by FDA, the labelling process begins to make it available in the market. Labelling process is all about development of prescribing information regarding the new drug. [3]

ix. Post-approval Research & Monitoring: While the drug is on the market, the drug developer company still needs to carry on post-approval monitoring. Through this monitoring, data regarding the drug's unpredicted serious side effects, modification of doses, and alternative usage can be obtained. [3]

2.1.3. In Silico Drug Discovery: From late twentieth to early twenty- first century, Drug discovery has gone through many changes. These changes were incorporated to anticipate whether a compound is going to be appropriate as a drug, or otherwise enable identification of unsuited molecules. Integration of in silico strategies emerged in this way. In Silico drug discovery strategies were developed for designing and optimizing the lead. It is supportive to the long-established, conventional methods (in vitro & in vivo) for drug designing. In silico studies totally depend on pre-existing large databases aided with high-efficiency screening functions and bioinformatics rules and formula to search, add notes providing explanations and comments alongside the data from a target's perspective. The chemo-informatics methods are also applied for lead design process. [4]

In general, drug designing is a process in which the main aim is to discover the new leads (i.e. the potential drugs) which have therapeutic benefits in diseased condition. In Silico drug discovery has become a faster process with the development of several computational tools and available large online databases containing information about three dimensional structures of different molecules. [5]

The two major in silico drug development methods are as follows-

a) Structure based drug designing- In this method predictions are made based on 3D structure of the target molecules. The major approach here is Molecular docking. It is a method which employs scoring functions along with various sampling algorithms. Docking can be executed in many ways based on the rigidity or flexibility of the ligand and receptor molecules. Another method to design drug is Hotspot grafting. It is mostly used in the cases where the structures of the binding protein as well as the target protein are accessible and the hot-spots on the interacting surface can be identified beforehand.

b) Ligand based drug designing- When information about the 3D structure of target molecule is unavailable, this method is used. Among other ligand based drug designing, two most common methods used in general, are Pharmacophore modelling and QSAR (quantitative structure-activity relationships). Pharmacophore modelling deals with only the explanation of crucial features of an active ligand. Q-S-A-R modelling controls the outcome of some specific properties on the ligand's activity.

There is another de novo approach to build new lead compounds with the use of fragments within the protein's active site. This process is known as Fragment based drug designing.

All the potential leads gained by different drug designing methods, must fulfil ADMET properties for being developed as an effective drug for a specific disease. With the help of in silico ADMET prediction tools the ADMET profiling has become a much faster process. Nowadays, there are many different types of software available for drug designing and ADMET property predictions. [5]

2.1.3.1. Merits of In-Silico Drug Discovery: Whereas in-vivo drug discovery techniques mandatorily take place inside the body of a whole live organism, in silico techniques are considered as far more practical and cost-effective experiments as they do not need live organisms for the experimentation process. Instead, these in silico models are simply some abstract representations modelling various human ailments. Moreover using computational methods can reduce the number of lab animals used in research works, ultimately supporting the rationale for constructing novel and safe potential drugs for diseases. [6]

The ever-rising costs and achievement of FDA approval for in-vitro/in-vivo drug development techniques are almost always issues of great concern. To eliminate these problems, innovative approaches are needed for better identification of drug targets and predictions of its efficacy against the specific disease under study. A solution to these problems in traditional way of drug development can be the use of in silico methods of drug designing techniques like molecular docking. Through the use of molecular docking we can rapidly screen the extensive libraries of ligands and targets; thus making this method more convenient one rather than the traditional ones. [6]

Computer-aided drug design (CADD) includes several different methods providing economical way-outs for identifying potential drugs. There are generally 2 broad categories of CADD methods- i) SB- CADD & ii) LB- CADD. The first one means

'structure' based and the later one means 'ligand' based ones. The plus point of using CADD method is that it gives a higher probability to detect and identify compounds with the desired properties. This in turn increases the possibility of a chemical substance to overcome the hurdles of pre-clinical tests. [6]

The clinical trials in traditional way of drug development can specify whether a product and/or a technology is unsafe or ineffective but they mainly fail to explain the cause that is making it unsafe or do not provide the way-out for overcoming it. Now, clinical trials done through in-silico mode offer simulations for the process of developing or evaluating medical instruments. In this way, the barriers of traditional clinical try-outs can be easily overcome by creating algorithm that identifies an error and also further simulates potential improvements. [6]

2.1.3.2. Demerits of In-Silico Drug Discovery: One main limitation of In Silico drug discovery is pharmacophore - LBDD. Here 'pharmacophore' indicates the specific part of a molecule, which is accountable for particular biological interplays & 'LBDD' means Ligand Based Drug Designing. Thus indicating the complex molecular dynamics that is faced here. This technique demands a lot of computation and depends on the very size of the simulated systems. The problem is the period of time, which is mostly very short (milliseconds to seconds) to analyse the folding of the whole protein. So this can be the cause of "inadequate sampling" for protein conformations. One of the main drawbacks with molecular docking is that we must ensure that appropriate scoring functions and algorithms are implemented; otherwise it can lead to defective screening of the molecules. [6]

2.1.3.3. Recent Trends of In Silico Drug Discovery: Nowadays, in-silico drug discovery techniques are not only used for new drug development, but they are also being used for drug repurposing as well.

An example is network-based drug-repurposing (NB-DRP). It is based on relationship between different biological compounds, organised into network format for identifying emerging properties at the level of a network. This established network helps to examine how cellular systems undergo various biological changes under different conditions. Structurally, a network is created by connecting the nodes in a graph, here each node represents a drug or a biological target. This is beneficial to identify rather

complex or complicated diseases which manifest due to the interactions among several biological networks. [6]

A study to model diagnosis progression in case of human disease, was carried out based on network-based analysis. A network of 'claims data' is constructed, containing characteristic accounts or records of different genetic as well as non-genetic human diseases, including the hazardous elements too. Additionally, this data-set is included in patients' medical records in a chronological order. The developed network based on these claims, allowed broad inspection of the links between diagnoses or progress of various diseases. This also provided a finer perception of the interrelated nature of relationship spanning over various illnesses. [6, 7]

In silico imaging in clinical trials are also being used to overcome the challenges of conventional clinical trials. Here computational modelling is used in biological sciences. It is basically computationally simulated form of a complete imaging system. The simulation does/makes all of the followings- source, objection, detection, interpretation; these are particularly used for the evaluation of a new technology. The evaluation of in silico imaging system is done through- detection, diagnosis, and monitoring of a disease for treatment. In silico imaging has further redefined R&D in imaging studies.

In silico clinical trials has requirements for 'virtual patients' (VPs). To create a virtual patient population, it is needed to make use of the 'Virtual Physiological Human' (VPH). The VPH is a collaborative system where many organizations equally share resources. These resources are used to comprise computer models mimicking biochemical, mechanical and physical roles of an alive body system for *Homo sapiens*. It is a European venture which offers joint and co-operative investigational, inquisitive research & study based on the entire body of Homo sapiens, even to the extent of genomes. 'VP's are formed by narrating the frameworks of the desired group within quantitative VPH prototypes. Then the prototypes are encoded using qualitative information based on human physiology under study. VPs are thus more beneficial than human volunteers. [8]

In silico medicine has great therapeutic response on virtual organs or systems. But for now, human individuals suffering from the particular disease under study, are still required for last-phase drug developing researches to investigate the finest model for

tolerability and efficacy study of the drug. In future in-silico type of trials will be swifter and more economical compared to the conventional methods. This will in turn reduce the total number of human participants in clinical trials. [6]

2.1.4. Breast Cancer: This type of cancer first manifests inside the breasts of a living human being. Cancer manifests firstly when normal body cells suddenly start to divide and grow in a rapid and uncontrollable way. At first, breast cancer cells generally form a lumpy tumour which can be observed by the use of X-ray internally or can be noticed as a ball of mass externally. Breast cancer is seen mostly in female human population, but males can also develop breast cancer. It is important to understand that in most cases breast lumps tend to be of benign type and not malignant i.e. they do not have potential to develop cancer. Non-malignant mammary gland tumours are just some unusual growths which never venture outside the breast (metastasis), but some benign tumours may increase the risk of developing cancer in future. Any new changes found in breasts (like a lump, redness or tenderness of breast) must never be ignored and needed a prompt check up by the doctor to identify the type of the tumour i.e. either benign or malignant, whether it might influence the patient's future risk of developing cancer or not. Several factors like lifestyle factors, habits, as well as inherited genes, can be the cause of breast cancer.

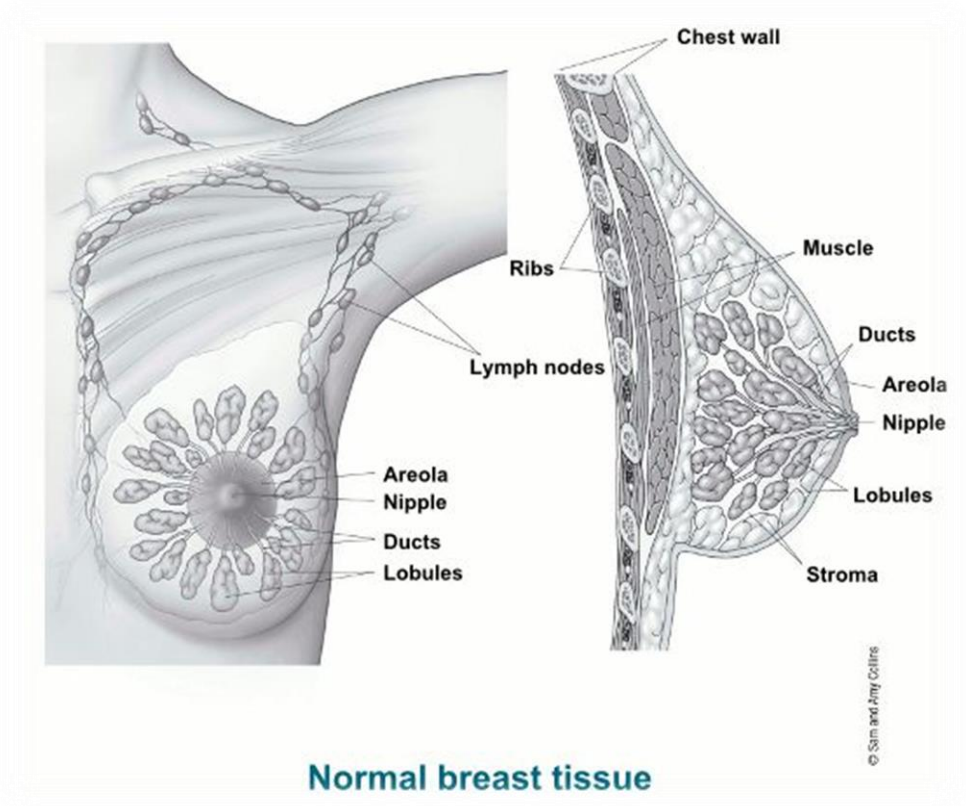

Fig.2.1. Different parts of the breast such as the ducts, lobules, and lymph nodes [12]

Breast cancers can start from different parts of a breast-

- When begins in the breast milk-carrying ducts, it is ductal cancer
- When starts at the breast milk making glands, it is lobular cancer
- Other less common types are like phyllodes tumor and angiosarcoma
- When starts in other tissues of breast, they are named as sarcomas and lymphomas but actually those are not considered as true form of cancers of breast.

Not all but in most cases, breast cancer starts as a lump formed in the breast. They are detected by mammograms; which identifies cancer at an early stage, generally before they can be noticed externally as lumps, and even prior to the development of cancerous symptoms. [9]

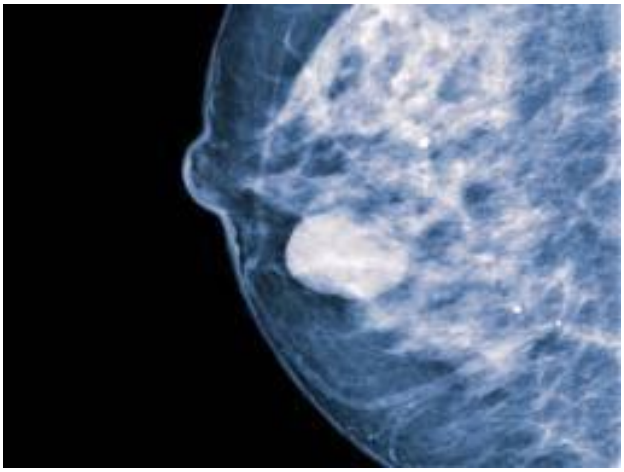

Fig.2.2. Mammography image of breast cancer [13]

Cancer is spread by cancerous cells going into the blood circulation or lymph circulation system and then carried along with the blood and lymph to different parts of the human body through blood vessels or lymphatic channels. The lymphatic vessels are generally employed to carry lymph fluid away from the breast towards the lymph nodes. Cancerous cells emerging from breasts could intrude into lymphatic vessels and travel to lymph nodes where they start to grow and divide. If cancer cells get spread to the lymph nodes, in most cases the cells also travel through the lymphatic system and get metastasized to different parts or organs. Thus finding cancer in even one lymph node can affect the overall scheme of treatment. Most of the cases, a surgery is needed to exclude a single or more lymph nodes in order to understand whether the cancer has spread to other parts or not. But there are exceptional cases too; not all

women with cancerous lymph-nodes develop metastasis, but in some cases females having no cancerous cells at all in their lymph-nodes may be prone to metastasis in later stages. [9]

2.1.4.1. Treatments for Breast Cancer: In broad sense, two types of treatments are mainly provided for the treatment of breast cancer cases-

A) **Local treatments**

Some treatments for breast cancers are considered local because they only treat the tumour in the affected part without affecting the patient's other body parts.

Most women affected by breast cancer generally go through surgery for the removal of the cancerous tumour. Depending on the breast cancer type and the degree of advancement of the disease, other types of treatment might be needed as well. This treatments are needed either before or after surgery, or sometimes it is needed for both the before and after breast cancer surgery.

- Surgery for Breast Cancer –
    a) Breast-conserving surgery: where cancer containing segment of the breast is excised;
    b) Mastectomy: where whole of one breast is excised;
    c) Double Mastectomy: both breasts are completely removed in severe conditions.
- Radiation for Breast Cancer-
    a) External beam radiation therapy: Here a system placed outside the patient's body uses focused radiation on the cancer affected part of the body. It is the most common type of external beam radiation therapy (EBRT) for women.
    b) Brachytherapy/ internal radiation therapy: There is another way to deliver radiation therapy. Here for a short time period, a tool in the form of a small radio-active pellet is placed inside the area of mammary gland tissue wherefrom the cancer had been removed previously; it is called the tumour bed.

B) Systemic treatments

Using drugs for treating breast cancer is called systemic therapy as those drugs are able to reach cancerous cells that are situated at any place inside the human body. They can be administered orally; or intramuscularly, or even intravenously i.e. directly injected into the bloodstream. Regarding the degree of advancement of the disease, the stage and type of breast cancer, various drug treatments are employed, those are:

- Chemo-therapy
- Hormonal Therapy
- Targeted Drug Therapy
- Immunotherapy

2.1.5. Drugs & Proteins involved in Breast Cancer:

There is a continuous search for effective medicines that might be able to diminish the risk of affliction of cancer in the mammary glands, especially for female human beings who generally fall at high risk category.

Receptors are some kind of proteins that are present on the surface of cells and can get attached to certain substances present in blood. Normal and cancerous mammary gland cells have certain receptors that get attached with hormones and depending on these hormones, the cancer cells grow rapidly.

Based on the breast cancer cells having one, both, or in some cases none of these receptors, they can be categorized into different types of breast cancer as the followings-

- **Estrogen receptor positive:** Cancer cells having estrogen receptors are known as ER-positive (written as ER+) cancers.
- **Progesterone receptor positive:** Cancerous cells having progesterone receptors are known as PR-positive (written as PR+) cancers.
- **HR-positive:** HR-positive means hormone-receptor positive i.e. having active hormone receptors on the cell surface. The cancer cells which have one or both of the hormone receptors (ER, PR) are called hormone-receptive positive or hormone positive (HR+) breast cancer.
- **HR-negative:** Some cancer cells can have neither the estrogen receptor nor the progesterone receptor on their cell surface; this type of cancer is called hormone-receptor negative or hormone-negative (HR-) breast cancer.

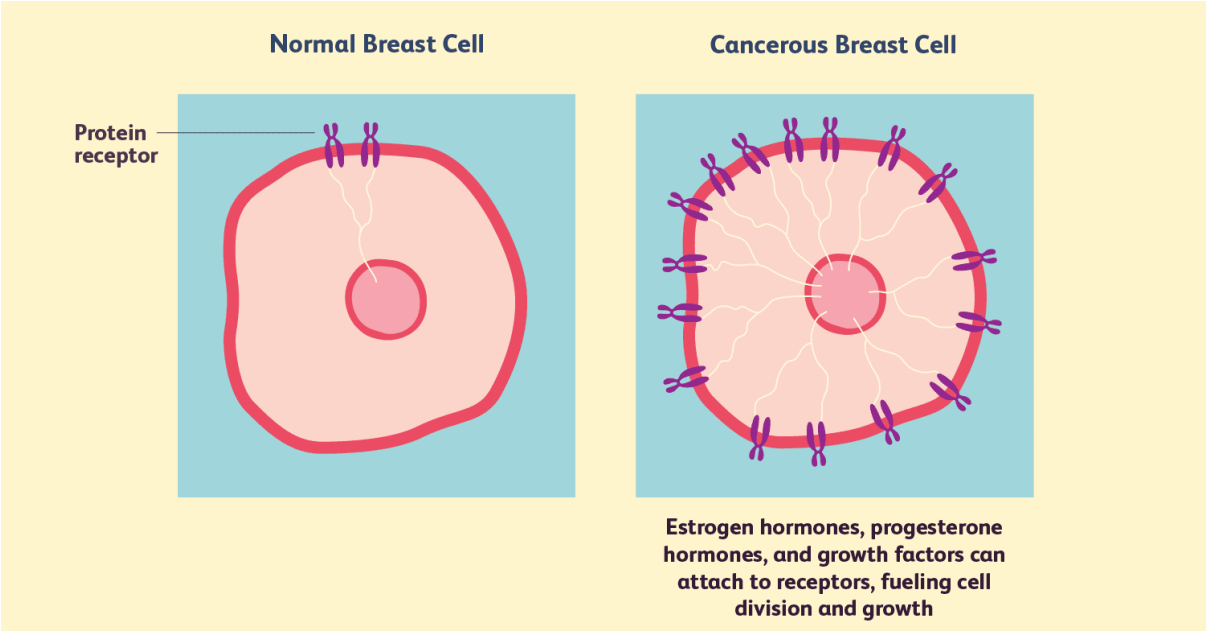

Fig.2.3. Hormone receptor status in breast cancer [14]

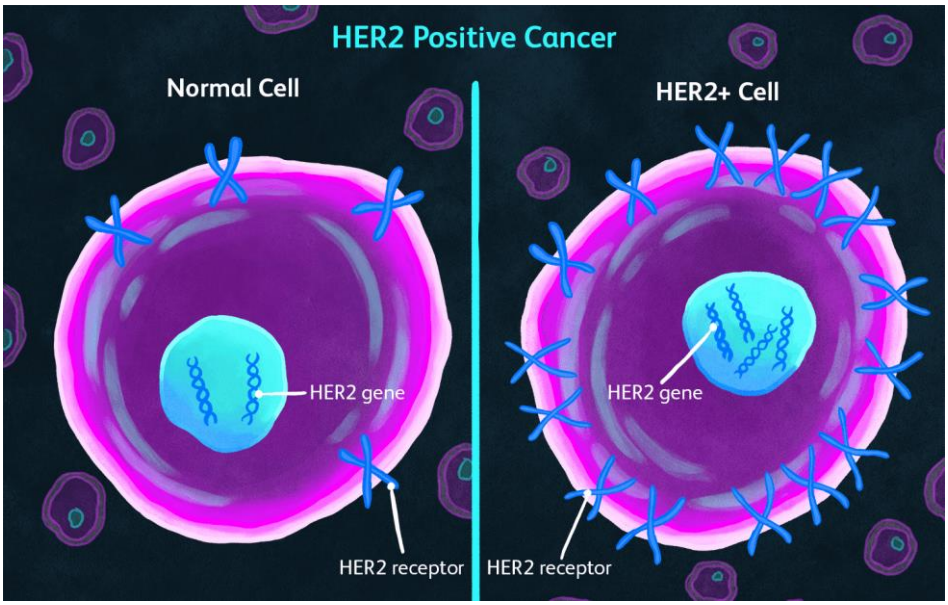

Fig.2.4. Normal breast cell vs. HER2+ cancerous breast cell [15]

- **HER2 receptor positive & negative: '**HER2' stands for 'Human Epidermal Growth Factor Receptor 2'. The role of HER2 in normal cells is to help control cell growth. Irrespective of being cancerous and noncancerous, all breast cells have HER2 receptors on their cell surface. The important differentiating factor between being normal or cancerous, is that HER2+ cancer cells in the mammary glands possess 40 - 100 times

more HER2 receptors on their cell surface than HER2-negative cells or normal mammary gland cells generally have in their possession.

Cancer cells that are HER2 negative generally grow more slowly than HER2+ cancer in breasts. In extremely rare cases, HER2-negative mammary gland cancer cells are seen to recur after proper breast cancer therapy is done or spread to other parts of the body; the chances are really low in comparison with cancer cells possessing a large number of HER2 receptor on their surface. In HER2 positive cases, the abundance of receptors fuels the cancer rapidly as they get stimulated by HER2-protein, assisting the rapid growth and spread of breast cancer cells.

- **Triple-Negative Breast Cancer (TNBC):** TNBC contributes to 10-15% of all mammary gland cancer cases; this term indicates that these type of cancerous cells neither possess ER or PR receptors nor do they make HER2 protein in excessive amount. This type of cancer cells test "negative" on all 3 tests. This is more common in women who are less than 40 years of age belonging from Afro-American society. Researchers don't yet know what the actual cause behind TNBC is, but they assume mutated BRCA1 gene might play a role here. The BRCA1 gene's role is to prevent cancer. When the genetic mutation happens, the gene's course of action is reversed, in turn making the cells more prone to cancer.

If estrogen and progesterone hormones cannot bind to the receptors, this may stop further progression of the cancer. There are certain drugs that can be used →

- The '*estrogen blocking drugs'* have their use in treating and preventing breast cancer. Examples are 'Tamoxifen' and 'Raloxifene' which are being used to treat ER+ cancer for a long time. Recent studies have shown that '*Aromatase Inhibitors'* drugs e.g. 'Exemestane' and 'Anastrozole' are also capable to prevent breast cancer.

- Other clinical trials based on '*non-hormonal drugs'* for reduction or prevention of human breast cancer, are also being conducted these days. Some of those drugs are 'metformin' (diabetes drug), 'ruxolitinib' (for blood and bone marrow diseases), and 'bexarotene' (T cell lymphoma).

- Triple Negative Breast cancer or TNBC can't get treated with hormone therapy or targeted therapy; treatment option is only chemotherapy. In general, TNBC respond well to initial chemotherapy, but it recurs more frequently than other cancers. These

cancers more commonly affect women who are less than 40 years old, mainly belonging from Afro-American society, or who possess mutated BRCA1 gene.

- In the year of 2019, the '*immunotherapy drug'*, 'Atezolizumab', got approval for usage along with the chemotherapeutic drug 'nab-paclitaxel' in treating women with advanced TNBC that makes the '*PD-L1' protein* a.k.a. 'Programmed death-ligand 1'. *PDL1*, the protein which helps to keep the body immune cells at check from attacking non-harmful cells in the body. Some cancer cells possessing high amounts of PDL1, enabling them to trick our body immune system; thus they can avoid being attacked even though they are harmful substances for the body. Other targets of novel drugs for fighting mammary-gland cancer, have been discovered over the last few years. Drugs have been also designed according to those targets, e.g. *kinase inhibitors*, are now under study to treat TNBC. For example *AKT inhibitor*, 'ipatasertib' when used along with 'paclitaxel', have shown very good results in treating TNBC as the first treatment. Another AKT inhibitor, 'capivasertib' also showed good effectivity when taken along with paclitaxel.

- Hormone (estrogen, progesterone) receptor tests are conducted on a regular interval for breast cancer cells to determine treatment options. About 60% of breast cancer cells also have androgen receptors (androgen being the male hormones). So '*Androgen receptors inhibitors'* are also used to prevent breast cancer. Studies in women affected by breast cancer have shown response when antiandrogen drug 'bicalutamide' is used to treat TNBC (triple-negative breast cancers) that has the androgen receptor. Prostate cancer has been treated using bicalutamide for many years. More advanced researches for breast cancer are still going on. [9]

2.1.6. Details of Drugs Used for Breast Cancer→

2.1.6.1. Drugs that Block Estrogen Receptors:

1. **Tamoxifen:** This drug is used to block estrogen-receptors on breast-cancer cell surface. It stops the hormone from attaching to the cancer cells, making them grow and divide. This drug acts like an anti-estrogen for breast, but in other tissues (uterus, bones) it acts like estrogen. Owing to the peculiar phenomenon, it is known as a '***selective estrogen receptor modulator' (SERM)***.

2. **Toremifene:** Another SERM whose working principle is same but its use is less common. This is only permitted to use in the treatment for postmenopausal women whose breast cancer has already metastasized. These drugs come in the form of oral pills.

3. **Fulvestrant:** Fulvestrant blocks as well as damages cell surface estrogen receptors. This drug functions similar to an anti-estrogen inside the body. It is called '***selective estrogen receptor degrader' (SERD)***. It is limited to be used in post-menopausal women. [9]

2.1.6.2. Drugs that Lower Estrogen Levels:

1. **Aromatase inhibitors (AIs):** These drugs prevent the production of estrogen. Prior to menopause, mostly estrogen is synthesized by the ovaries. But for women with nonfunctional ovaries (menopause, specific therapies) a little quantity of estrogen yet gets generated in the adipose tissue by aromatase enzyme. AIs block aromatase and inhibit production of estrogen. Examples of some effective AIs are- 'Letrozole', 'Anastrozole', & 'Exemestane'. These drugs are produced in the form of pills and taken daily by the patients.

2.1.6.3. Drugs for Ovarian Suppression:

1. **LH-releasing hormone (LHRH) analogues:** These are used instead of oophorectomy i.e. the surgical removal of ovaries which are the main source of estrogen. They block the estrogen making signal from reaching the ovaries, in turn causing menopause temporarily. E.g. 'goserelin' and 'leuprolide' can be administered singularly or in combination with other hormonal drugs (tamoxifen, aromatase inhibitors, fulvestrant). This is considered as a hormonal therapy for pre-menopausal female human beings. [9]

2.1.6.4. Drugs for HER2+ Breast Cancer:

Approximately in 1 among 5 women with breast cancer, it is seen that the cancer cells have excessive amount of growth-promoting surface protein, HER2 than that is present in the normal cells. These HER2-positive breast cancers are of more aggressive type. Different drugs have been designed to aim for the HER2 protein over the years. Those are as follows-

A) <u>Monoclonal antibodies</u> are man-made immunoglobulins or immune system proteins that are specifically crafted in laboratories, to bind to HER2 protein on the cell surface, and stop the cancerous cells from growing.

1. **Trastuzumab:** Not only early-stage but also advanced breast cancer can be treated. This drug can be given associated with a chemo or given alone. It is given intravenously (IV).

2. **Pertuzumab:** It can be administered in combination with trastuzumab and a chemo for treating early or advanced mammary gland cancer. This drug is given as IV.

3. **Margetuximab:** This is utilized in combination with chemo to treat advanced stage breast cancers. This drug is only administered to the patient after at least two other drugs targeting HER2 have been tested out previously. This drug is administered through vein. [9]

B) <u>Antibody- Drug conjugates (ADC)</u>: An ADC is formed by a monoclonal immunoglobulin joined with a chemotherapeutic drug. Here anti-HER2 antibody carrying the chemo along with it gets attached to the HER2 protein receptor on cancer cells.

1. **Ado-trastuzumab emtansine:** To treat early-stage breast cancer after surgery or advanced breast cancer in women who have already been prescribed with trastuzumab and chemo, this drug is administered intravenously.

2. **Fam-trastuzumab deruxtecan:** Another ADC that can be used alone to treat breast cancer which is untreatable by surgery or that has metastasized, normally after other anti-HER2 targeted drugs have been tried out. It is given intravenously. [9]

C) <u>Kinase Inhibitors</u>: HER2 is a kinase protein. Kinases relay signals instructing the cells to grow or divide. Drugs which block kinases activity are called kinase inhibitors.

1. **Lapatinib:** This drug comes in the form of pill which can be consumed daily. It treats advanced mammary gland cancer, in combination with other chemo or hormonal therapy drugs.

2. **Neratinib:** Another 'kinase-inhibitor' pill which is taken daily for a length of 1 year. It is effective for the treatment of early-phase breast cancer after the patient has gone through 1 year course of trastuzumab. This can be given in combination with chemo drug to treat metastatic disease, after other anti-HER2 targeted drugs have been tried on the patient.

3. **Tucatinib:** This one is taken as oral pills as well, normally two times a day. This drug is useful for treating patients in the advanced stage of breast cancer, after other anti-HER2 drugs have been administered on the patient. It is used in combination with trastuzumab and chemo drug, capecitabine. [9]

2.1.6.5. Drugs for Hormone Receptor-Positive Breast Cancer:

In every 3 mammary gland cancers, 2 of them are found to be HR-positive (ER+ or PR+).

In these scenarios hormone therapy majorly helps in treating the cancer.

Use of targeted therapy drugs can in turn be helpful to make hormone therapy even more effective for cancer patients, but the targeted drugs can also give rise to side effects.

1. **CDK4/CDK6 inhibitor drugs: Palbociclib, ribociclib,** and **abemaciclib** drugs have the ability to block cyclin-dependent kinase (CDKs) proteins, specifically CDK4 and CDK6. In HR+ breast cancer cells blocking these proteins prevents the cells from dividing as a consequence. As the result it slows down the cancer growth. These drugs have got approved to be used in cases of advanced HR-positive, HER2-negative breast cancer. These are taken as oral pills for one to two times a day.
2. **mTOR inhibitor drugs: Everolimus** is a target oriented drug called as the inhibitor of mTOR. It is used to block mTOR protein present in cells helping them to grow and divide. This drug stops tumours from sprouting new blood vessels, limiting their growth. This drug also helps hormone therapy drugs to work better. It is generally prescribed to post-menopausal women and who suffer from advanced HR-positive, HER2- negative breast cancer. This drug comes as a pill, taken once a day.
3. **PI3K inhibitor drugs: Alpelisib** is another target oriented drug that falls under PI3K inhibitor group of drugs. It blocks one type of PI3K protein present in cancerous cells, which leads to stop the cells from further dividing

and growing. This drug is prescribed in combination with fulvestrant to treat post-menopausal females having advanced HR-positive, HER2-negative breast cancer along with a mutated PIK3CA gene which is a result of aromatase inhibitor treatment. Almost 30% to 40% of breast cancers have a PIK3CA genetic mutation. The physician tests the patient's blood or tumour for the signs of this specific genetic mutation before initiating the treatment process using this particular drug. This drug comes as oral pills, usually consumed one time per day. [9]

2.1.6.6. <u>Drugs for women with BRCA gene mutation</u>:

1. **Olaparib** and **talazoparib** drugs are called '*PARP-inhibitors'*. 'Poly Adenosine diphosphate-Ribose Polymerase' proteins are enzymes that help to repair damages of DNA inside our body cells. The BRCA1 and BRCA2 genes also help to repair our damaged DNAs but they do it in a slightly different process. Mutations in one of the above mentioned genes in turn stalls the natural DNA repair process. PARP inhibitors actually block the PARP proteins. They stops the cancer cells from repairing and let them die in the process. PARP inhibitors only target cancerous cells and do not affect normal cells in the patient's body. Tumour cells having a mutation in BRCA gene naturally face difficulty in mending corrupted DNAs moreover, blocking of PARP can result in the demise of the cells. Above-mentioned two drugs are administered to treat metastatic, HER2-negative mammary gland cancer in patients having mutated BRCA gene along with a previous history of chemotherapeutic treatment. Olaparib is prescribed for patients who have had hormonal therapy when the cancer is of HR-positive type. A very few number of cases have been found to have inborn BRCA genetic mutation, present in all the cells of the body. All of these drugs come in the form of oral pills and are consumed two times per day. [9]

2.1.6.7. <u>Drugs for TNBC</u>:

In triple-negative mammary-gland cancer, the speciality of the cancerous cells is that they don't have ER and/or PRs; they don't even produce excessive amount of HER2 protein.

1. **Sacituzumab govitecan:** In this particular ADC, the monoclonal immunoglobulin part binds with Trop-2 protein present on mammary-gland

cancer cells; the chemo is brought directly to those cells. Some of the breast cancer cells have excess amount of Trop-2 which helps them to grow and spread rapidly. After other chemotherapeutic drugs have been tried out with the patient, this ADC may be administered alone to treat advanced TNBC. This drug is administered as IV in a weekly manner; 2 weeks at first, followed by a gap of one week, and after that resumed again. [9]

2.1.7. "*Avicennia marina*"- The Plant under Study

Scientific classification: [10, 11]

Domain – Eukaryota

Kingdom – Plantae

Phylum – Spermatophyta

Subphylum – Angiospermae

Class – Dicotyledonae

Order – Lamiales

Family – Acanthaceae

Genus – *Avicennia* Species – *marina*

Fig.2.5. The grey mangrove, Avicennia marina [16]

Fig.2.6. Excreted salt on the underside of a Avicennia marina leaf [18]

Avicennia marina is one of the major species of mangrove which belongs to the Acanthaceae plant family. This plant is called grey mangrove or white mangrove in common tongue. Like other mangroves, this is a small tree and grows in coastal saline or brackish waters. This mangrove generally develops as a tree with a height ranging from 9.8 to 32.8 ft. In tropical regions, they can grow up to 46 ft. A gnarled arrangement of multiple branches is generally seen in this mangrove plant. The bark is smooth and light-grey in colour, consisting of thin, and brittle flakes. This can be greyish white in coloration, indicator of the origin of its common name.

Leaves are very thick, around 5 to 8 cm. in length. The colour of the leaves is bright and lustrous green on the upper part, and silver-white, or greyish, with minute hairs on the surface below. These

plants secrete excess salt and are considered to be highly salt-tolerant mangrove plant species. *A. marina* generally grows best in 75% seawater and can tolerate >250% seawater. It is one of the rare plants that excrete about 40% of salt through its salt glands present in the leaves. It has an extraordinary type of roots that can exclude the salts in water from entering the plant body. [17]

Fig.2.7. Flower of A. marina [18]

It has aerial roots i.e. the pneumatophores which can reach a height of about 20 centimetres. The diameter of the pneumatophores is 1 cm. in general. All the pneumatophores enable the tree to absorb oxygen from the air, which is deficient in its habitat soil.The coloration of the flowers ranges from whitish to gold-yellow. Flowers are less than 1 cm across, and they are found as a cluster of three to five in general. The fruit has large cotyledons. They produce a big, fleshy seed, mostly seen to be germinating while still on the tree and falls down on the soil as a seedling. This mangrove plant can be of stunted height if it grows in too saline waters, but can grow to its full height in water condition where traces of both salt and fresh water are present side by side. This tree can tolerate high salinity as they excrete the extra salts through their leaves.This is a highly variable mangrove plant having several ecotypes, and their forms closely resemble the other *Avicennia* species.

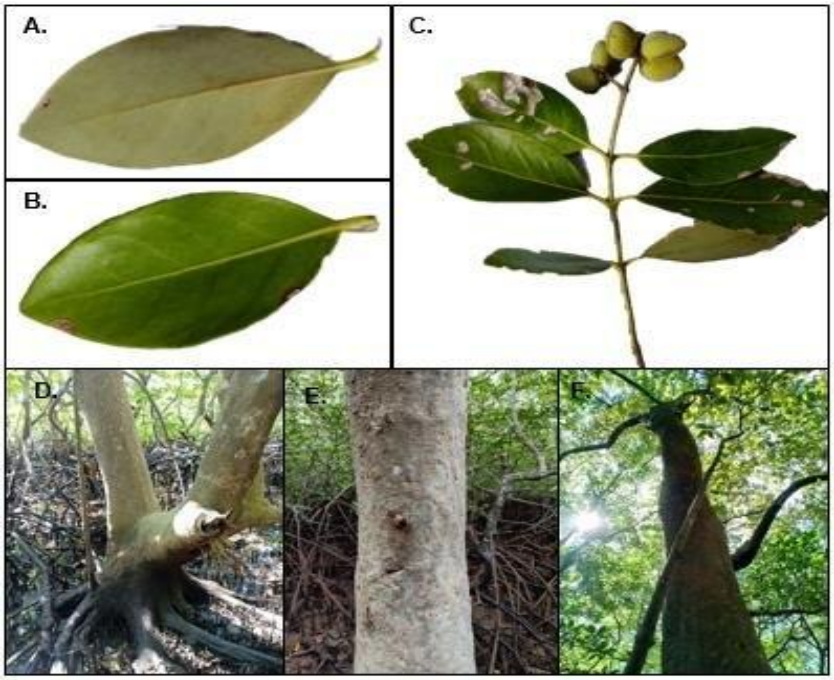

Fig.2.8. Different plant parts of A. marina: A. Leaf lower surface (grey-yellow); B. Leaf upper surface (green & glossy); C. Arrangement of leaf (opposite); D. Roots (pneumatophores); E. Trunk (smooth bark with lenticels); F. Tree top. *[19]*

It is seen to bear extreme conditions like high winds, different pests and various diseases. *A. marina* is considered to be a pioneer species in muddy soil (pH 6.5 - 8.0), although it cannot grow in the shades. As of now there are three recognized subspecies: 1) A. m. subsp. Australasica, 2) A. m. subsp. Eucalyptifolia, 3) A. m. subsp. marina. [11] It is found in large parts of Africa and Asia specifically in South and south-east part of Asia along with Australia, and New Zealand. [11]

Fig.2.9. Distribution of mangrove forest sites in India; natural habitat of A. marina plant

Table – 2.1. *Major Phytoconstituents found in A. marina plant's leaf*

Main Groups	***Sub-groups***
Flavonoids	Luteolin 7-*O*-methylether 1 [20]
	7 -o-methylether 2 [20]
	Isorhamnetin 3-*O*-rutinoside [20]
	Chrysoeriol 7-Oglucoside [20]
	Luteolin 7-*O*-methylether 3'-*O*-β-D-glucoside [20]
	5-hydroxy-4; 7-dimethoxyflavone [21]
	Quercetin [21]
	Kaempferol [21]
	4'5-dihydroxy-3'-5,7-diimethoxyflavone [22]
	4',5-dihydroxy-3',7-trimethoxyflavone [22]
	4',5,7-trihydroxyflavone [22]
	3',4',5-trihydroxy-7-methoxyflavone [22]
Steroids	ß-sitosterol [21]
	Ergost-6,22-diene-5,8-epidioxy-3ß-ol [21]
Terpenoids	Lupeol [21] [23] [24]
	Betulin [21] [24] [25] [26]
	α-amyrin [27]

Main Groups	*Sub-groups*
Fatty Acids	Oleic acid [28] [29]
	Linolenic acid [29]
	Palmetic acid [29]
	Stearic acid [29]
	Lauric acid [29]
	Myristic acid [29]
Glucosides	geniposidic acid [30]
	2'-cinnamoyl-mussaenosidic acid [30]
	Mussaenoside [30]
	2'-cinnamoyl-mussaenoside [30]
	10-*O*-5-phenyl-2,4-pentadienoyl-geniposide [30]
	7-*O*-5-phenyl-2,4-pentadienoyl-8-epiloganin [30]
	10-*O*-[E-cinnamoyl]-geniposidic acid [31]
	10- *O*-[E-*p*-coUmamaheswarraoroyl]-geniposidic acid [32]
	10-*O*-[E- caffeoyl]-geniposidic acid [32]
	2'-*O*-[E-cinnamoyl]mussaenosidic acid [32]
	2'-*O*-[2E,4E-5-phenylpenta-2,4-dienoyl]mussaenosidic acid [32]
	2'-*O*-4 mehtoxycinnamoylmussaenosidic acid [32]
	2'-*O*-coUmamaheswarraoroylmussaenosidic acid [32]
	Marinoids A – E [33]
	Verbascoside [34]
	Isoverbascoside [34]
	Derhamnosylverbascosid [34]
Others	*p*-methoxy cinnamic acid [21]
	R-hydroxy-5-phenyl-4E-pentanoic acid [33]
	Syringaresinol [33]
	Indolyl-3-arboxylic acid [34]

2.2. Literature Review:

Since a very long time mangrove plants have been known as very useful against many diseases. [35] They are potent sources of bio-active compounds e.g. antioxidant, anti-diarrheal, anti-inflammation, anti-diabetic and anticancer compounds. Plant based natural products having antitumor properties are divided into 12 distinct chemical groups. Those groups are as follows- 1) alkaloids, 2) terpenoids, 3) phenylpropanoids, 4) lignans, 5) glycosides, 6) aldehydes, 7) lipids, 8) unsaponified lipids, 9) proteins, 10) polysaccharides, 11) nucleic acids and 12) unidentified compounds. The secondary metabolites of plants have been used as medicines from ancient era. Alkaloids, phenolic compounds, steroids, terpenoids etc. are products of secondary metabolism and these chemical compounds have pharmacological importance which can be used to design potential drugs. Almost 60% of drugs approved for treating cancer have natural origins. There are some well-known plant-derived anticancer drugs, vincristine and vinblastine falling in the category of Vinca alkaloids; original source of these alkaloids is a flowering plant named Madagascar periwinkle (the scientific name is *Catharanthus roseus*). This little plant has anticancer properties. Triterpene, betulinic acid compounds isolated from mangrove plant *Avicennia officinalis* have anticancer properties and are seen effective against Human leukaemic cell line (HL 60). It has been shown through different studies that bioactive compounds like Naphthoquinones, avicequinone, iridoid glycosides isolated from *Avicennia marina* have potential cytotoxic and antitumor peoperties. [35]

According to the findings of a recent research work, it can be said that leaves of A. marina mangrove plant can inhibit uncontrolled growth and division of breast cancer cell lines. It was found by the research study that 'luteolin' is the most active compound among other phytochemical constituents in crude methanol extract of A. marina. The 50% cell cytotoxic concentration (CC50) of crude methanol extract of A. marina was 250 µg/ml and the concentration of luteolin was 28 µg/ml. This compound acted as an apoptotic agent against MDA-MB 231 cells, which causes the DNA fragmentation of the cancerous cells. The level of mRNA expressed in Bcl-2 and p53 was found to be significantly decreased and increased respectively in cancerous cells treated by luteolin. Comparing the ultimate results it can be said that Luteolin isolated from Avicennia marina has high probability to initiate apoptosis on mammary gland cancer cell line by controlling p53 and Bcl-2 pathways. [36]

Through another study it was found that A. marina mangrove plant extract was definitely more active against the bacterial pathogens compared to its activity against yeasts. The antibiotic

effect was seen as diverse bioactive secondary metabolites are present in the plant body. The chemical profile of the methanolic extract of the plant was done through GC-MS. The gas chromatography-mass spectrometry results showed that the main phytochemical constituents were the followings- benzene-ethanol, 4-hydroxy-(RT = 12.173), followed by benzaldehyde, 3- methyl- (RT = 6.811). In the end the GC-MS data confirmed that anti-microbial activity of A. marina extract was either the result of the collective effect by all phytochemical constituents or the combined activity of major phytochemicals only. So it can be said that the mangrove Avicennia marina has antibacterial and anti-candidal potency as well. [37]

Another study has shown that biosynthesized silver nanoparticles (AgNPs) from A. marina mangrove have anti-cancer activity against A549 lung cancer cell line by inducing ROS/mitochondrial damages. The biosynthesized silver nanoparticles were crafted using mangrove plan's extract. The resultant Ag-NPs were determined by many different physio-chemical characterizations like UV-spectrometry and XRD analysis. The shape of the newly formed Ag-NPs was morphologically identified by Scanning Electron Microscopy and Transmission Electron Microscopy respectively. The anti-cancerous characteristic of manufactured Ag-NPs was seen at 50 µg/mL conc. against A549 lung cancer cells with the help of MTT assay and the ability to fuel ROS generation and mitochondrial membrane at IC50 conc. of silver nanoparticles were also established by the use of fluorescence microscopy with 2′,7′-Dichlorofluorescein diacetate (Dcfh-DA) and rhodamine 123 dyes respectively. The conclusion was that the synthesized Ag NPs has promising anti-cancer properties and effective against A549 lung cancer cells. [38]

A study in 2021 has shown that Avicennia marina plant extracts have anticancer and anti-proliferative activity. To discover the relationship between composition and function of crude hexane *A. marina* leaf extract, this study concentrated on investigating the anti-proliferative, cytotoxic and apoptotic effects on three particular cancer cell lines- HCT-116, HepG2 & MCF-7, which originally belong to the classes of colon, liver and breast cancer cell lines respectively. The phytochemical-constituents of the hexane leaf extract were detected using liquid chromatography-mass spectrometry (LC-MS) method. The result showed that the leaf extract showed a weak initiation of apoptosis. The apoptotic bodies were formed. It also showed a high cell inhibitory impact on all of the test cell lines with certain IC50 values. Moreover it was able to inhibit cell cycle at G0/G1 stage for HCT-116. It was also observed that in case of HepG2, and MCF-7 cell cycle was stopped at S phase. Acc. to these results, it was concluded that

hexane extract of *A. marina* leaves can be considered for further new anti-cancer drug designing researches. [39]

Another recent study has shown that ethanol extract of *A. marina* mangrove plant leaves gone through maceration till 48 hours exhibited highest anti-oxidant activities with IC50 value of 82.279 µg/mL. [40]

Through another study it has been shown that A. marina leaf extracts are rich in polyphenol content. Thus they are able to initiate apoptosis in human mammary gland and liver cancer cells as well. Experiments were done in the following breast cancer cell lines of human beings- BT483, MDA-MB-231, and AU565. The liver cancer cell lines of *Homo sapiens*, HepG2 and Huh7 and another normal human fibroblast cell line NIH3T3 were also included in the experiment. The chemotherapeutic potentials of *A. marina* plant extracts were tested for evaluation in a xenograft mouse model. The result showed that A. marina (ethyl acetate) EtOAc leaf extracts contain highest amount of flavonoid and phenolic constituents; exhibited most of the anti-cancerous activities too. Ethyl acetate leaf extract of A. marina inhibited growth of xenograft MDA-MB-231 tumour in a nude mice. This in turn suggests that ethyl acetate extract of A. marina leaves might give rise to a very effective treatment for breast cancer cases in human too. [41]

References:

1. Essentials of Medical Pharmacology (Seventh Edition) by KD TRIPATHI MD, Ex-Director-Professor and Head of Pharmacology Maulana Azad Medical College and associated LN and GB Pant Hospitals, New Delhi, India; Published by Jaypee Brothers Medical Publishers (P) Ltd.

2. https://www.genomind.com/360/an-introduction-to-pharmacokinetics-four-steps-of-pharmacokinetics

3. https://www.taconic.com/taconic-insights/quality/drug-development-process.html

4. Applications and Limitations of In Silico models in Drug Discovery- Ahamet Sacan, Sean Ekins, and Sandhya Kortagere

5. Sneha Rai et. al., Recent Trends in Computer Aided Drug Discovery. IJCB. 2016; Volume 5 (Issue 1): Page 54-76.

6. https://proventainternational.com/biology/a-critical-evaluation-of-the-advantagesand-limitations-of-in-silico-methods-in-clinical-research/

7. Jeong, E., Ko, K., Oh, S. et al. Network-based analysis of diagnosis progression patterns using claims data. Sci Rep 7, 15561 (2017). https://doi.org/10.1038/s41598- 017-15647-4

8. https://royalsocietypublishing.org/doi/10.1098/rsfs.2017.0067

9. https://www.cancer.org/cancer/breast-cancer.html

10. https://www.cabi.org/isc/datasheet/8074

11. https://en.wikipedia.org/wiki/Avicennia_marina

12. https://www.cancer.org/content/dam/cancer-org/images/illustrations/medical-illustrations/en/breast-cancer-images/normal-breast-tissue.gif/jcr:content/renditions/cq5dam.thumbnail.980.980.jpeg

13.https://assets.sutori.com/user-uploads/image/edc4627a-7118-4d1b-bfd9-3ef3088e1d57/20df3c94eed4827e577c7d2e4f2a1842.jpeg

14.https://www.verywellhealth.com/thmb/v5MxbiDmm92_gc1Pfs_mLc_too0=/614x0/filters:no_upscale():max_bytes(150000):strip_icc():format(webp)/hormone-receptor-status-and-diagnosis-430106-color-V12-c330f4d3c9d043aa8b081d2021f3623e.png

15.https://www.verywellhealth.com/thmb/E3CMmKKVwUM22O1ZSXLNf-eZ7ic=/614x0/filters:no_upscale():max_bytes(150000):strip_icc():format(webp)/diagnosis-and-testing-for-her2-positive-breast-cancer-4151804-53c6bdb1bb654ed69ce7f967626b7e4e.png

16.https://upload.wikimedia.org/wikipedia/commons/b/b5/Mature_mangrove_tree_%28Avicennia_marina%29_at_edge_of_Lake_Be_Malae.jpg

17. https://empowerias.com/blog/prelims-special-facts/avicennia-marina-empower-ias

18. https://en.wikipedia.org/wiki/Avicennia_marina

19. Mariano, Habagat & Dagoc, Frandel Louis & Dagoc, S & Espra, Aileen & Amparado, Ruben. (2019). Mangrove diversity, taxonomic classification, and morphological characteristics of natural and reforested mangrove forests in selected municipalities of Zamboanga Del Sur, Mindanao Island, Philippines. Journal of Biodiversity and Environmental Sciences. 15. 86-89.

20. Sharaf M, El-Ansari MA, Saleh NAM. 2000. New flavonoids from *Avicennia marina*. Fitoterapia. 71:274–277. doi: 10.1016/S0367-326X(99)00169-0

21. Jia R, Guo YW, Hou HX. 2004. Studies on the chemical constituents from leaves of *Avicennia marina*. Chin J Nat Med. 2:16–19.

22. Feng Y, Li XM, Wang BG. 2007. Chemical constituents in aerial parts of mangrove plant *Avicennia marina*. Chin Trad Herb Drugs. 38:1301–1303.

23. Mahera SA, Ahmad VU, Saifullah SM, Mohammad FV, Ambreen K. 2011. Steroids and triperpenoids from grey mangrove *Avicennia marina*. Pak J Bot. 43:1417–1422.

24. Khanh LP, Truc H, Kim NTT, Phung NKP, Thu NTH. 2013. Chemical constituents from leaves of *Avicennia lanata* non ridley, Phamhoang (Avicenniaceae). Sci Tech Dev. 16:20–25.

25. Ramanjaneyulu MVV, Venkateswara RB, Ramanjaneyulu K, Suvarna RP. 2015. Phytochemical analysis of *Avicennia Officinalis* of Krishna Estuary. J Pharm Drug. 3:176–180.

26. Majumdar SG, Patra G. 1979. Chemical investigation of some mangrove species, part I genus *Avicennia*. J Indian Chem Soc. 56:111–113.

27. Mahera SA, Saifullah SM, Ahmad VU, Mohammad FV. 2013. Phytochemical studies on mangrove *Avicennia marina*. Pak J Bot. 45:2093–2094.

28. Chinnappan RS, Natarajan SB, Kathiresan K, Sekar A, Sahu SK, Kuma A. 2013. Effect of bioactive substances from mangroves on anti-oxidant, anti- bacterial activity and molecular docking study against lung and oral cancer. Photon. 139:226–236.

29. Manilal A, Sujith S, Kiran GS, Selvin J, Shakir C. 2009. Biopotentials of mangroves collected from the southwest coast of India. Global J Biotech Biochem. 4:59–65.

30. Konig G, Rimpler H. 1985. Iridoid glucosides in *Avicennia marina*. Phytochemistry. 24:1245–1248. doi: 10.1016/S0031-9422(00)81110-3

31. Shaker, K. H., Elgamal, M. H. A., & Seifert, K. (2001). Iridoids from Avicennia marina. *Zeitschrift für Naturforschung C*, *56*(11-12), 965-968.

32. Feng Y, Li XM, Duan XJ, Wang BG. 2006. A new acylated iridoid glucoside from *Avicennia marina*. Chin Chem Lett. 17:1201–1204.

33. Sun Y, Ouyang J, Deng ZW, Li QS, Lin WH. 2008. Structure elucidation of five new iridoid glucosides from the leaves of *Avicennia marina*. Magn Reson Chem. 46:638–642. doi: 10.1002/mrc.2224

34. Fauvel MT, Taoubi K, Gleye J, Fouraste I. 1993. Phenylpropanoid glycosides from *Avicennia marina*. Planta Med. 59:955–981. doi: 10.1055/s-2006-959711

35. Das, G., Gouda, S., Mohanta, Y.K. and Patra, J.K., 2015. Mangrove plants: A potential source for anticancer drugs.

36. Momtazi-Borojeni, A. A., Behbahani, M., & Sadeghi-Aliabadi, H. (2013). Antiproliferative activity and apoptosis induction of crude extract and fractions of avicennia marina. *Iranian journal of basic medical sciences*, *16*(11), 1203–1208.

37. Aseer Manilal, Tsegaye Tsalla, Zerihun Zerdo, Gemechu Ameya, Behailu Merdekios, Shiju Easo John, Evaluating the antibacterial and anticandidal potency of mangrove, Avicennia marina, Asian Pacific Journal of Tropical Disease, Volume 6, Issue 2, 2016, Pages 136-140, ISSN 2222-1808, https://doi.org/10.1016/S2222-1808(15)60999-9.

38. Tian, S., Saravanan, K., Mothana, R. A., Ramachandran, G., Rajivgandhi, G., & Manoharan, N. (2020). Anti-cancer activity of biosynthesized silver nanoparticles using *Avicennia marina* against A549 lung cancer cells through ROS/mitochondrial damages. *Saudi journal of biological sciences*, *27*(11), 3018–3024. https://doi.org/10.1016/j.sjbs.2020.08.029

39. Albinhassan TH, Saleh KA, Barhoumi Z, Alshehri MA, Al-Ghazzawi1 AM. Anticancer, anti-proliferative activity of *Avicennia marina* plant extracts. J Can Res Ther 2021;17:879-86

40. https://aip.scitation.org/doi/abs/10.1063/1.5064126 Phytochemical contents and antioxidant activities of mangrove (Avicennia marina) leaves extract; AIP Conference Proceedings 2023, 020129 (2018); https://doi.org/10.1063/1.5064126 N. D. Takarina1,a) , G. A. F. Arif2 , and S. A. Juhriah3

41. Huang, C., Lu, C. K., Tu, M. C., Chang, J. H., Chen, Y. J., Tu, Y. H., & Huang, H. C. (2016). Polyphenol-rich Avicennia marina leaf extracts induce apoptosis in human breast and liver cancer cells and in a nude mouse xenograft model. *Oncotarget*, *7*(24), 35874–35893. https://doi.org/10.18632/oncotarget.8624

Chapter 3

Objectives & Proposed Plan of Work

3.1. Objectives:

Avicennia marina was chosen as a plant to be studied as it has its name in the pages of history i.e. it is considered to be a very ancient plant. This plant is well-renowned and it has been used since ancient era in traditional and folk medicine making. *A. marina* is a very old plant of origin that has gone through evolution over centuries and acquired the ability to get adapted in diverse climates. This tree is a reservoir of important phytochemicals which have the potential to be used for designing of drugs only after careful scientific studies. It is reported that *A. marina* plants exhibit many important pharmacological activities. Those are as follows- anti-diabetic, anti-cancer, anti-microbial, and many other beneficial activities are also seen. [1] Majority of cancer deaths in women in India have been attributed to breast cancer. Though better medications are available for breast cancer than other cancers nowadays still, current drug therapies have many side effects. Therefore, using antineoplastic (anticancer) agents from a natural source like medicinal plants may reduce the adverse effects on the healthy cells. Moreover using anticancer agents from plant as an alternative method of treatment is much more cost effective than the ongoing cancer treatments. The anticancer effect of A. marina plant extract is already reported against human promyelocytic leukemia HL- 60 cells [2], A549 lung cancer cells [3], WiDr colon cancer cells [4]. Methanol leaf extract of *Avicennia alba*, another species of Avicennia plant exhibited potent cytotoxicity against Human breast-adeno carcinoma i.e. MCF7 cell lines and HeLa cell lines (Henrietta's cancer cells). [5] In most of the previous studies based on A. marina plant, the whole plant was taken under consideration to carry out research works but at present we have selected the mangrove leaves only due to its abundance & ready availability; all of our experiments were conducted specifically using the A. marina leaf extract. The main goal of the current research work is the evaluation of the anticancer potential of *A. marina* leaf extract as well as to conduct the in-silico study of A. marina leaf phytochemicals against one of the human breast cancer responsible proteins, HER2.

3.2. Proposed Plan of work:

- The initial work was to collect and prepare *Avicennia marina* whole leaf crude extract.
- Qualitative and quantitative phytochemical analysis of *Avicennia marina* whole leaf extract was evaluated.
- Preparation of different concentration of *Avicennia marina* sample using lyophilized *Avicennia marina* crude leaf extract powder.
- Percentage of free radical activity of *Avicennia marina* leaf extract was calculated using DPPH method.
- Cytotoxic effect of different concentration of *Avicennia marina* sample was studied on normal, healthy mouse fibroblast cell (L929 cell line) by performing MTT assay.
- Anticancer potential of *Avicennia marina* leaf phytochemicals were carried out using in-silico docking technique.

References:

[1] Leen M. ElDohaji, Alshaimaa M. Hamoda, Rania Hamdy, Sameh S.M. Soliman, Avicennia marina a natural reservoir of phytopharmaceuticals: Curative power and platform of medicines, Journal of Ethnopharmacology, Volume 263, 2020, 113179, ISSN 0378-8741, https://doi.org/10.1016/j.jep.2020.113179.

[2] Karami, L., Majd, A., Mehrabian, S., Nabiuni, M., Salehi, M. and Irian, S., 2012. Antimutagenic and anticancer effects of Avicennia marina leaf extract on Salmonella typhimurium TA100 bacterium and human promyelocytic leukaemia HL-60 cells. *Sci Asia*, *38*(3), pp.349-55.

[3] Tian, S., Saravanan, K., Mothana, R.A., Ramachandran, G., Rajivgandhi, G. and Manoharan, N., 2020. Anti-cancer activity of biosynthesized silver nanoparticles using Avicennia marina against A549 lung cancer cells through ROS/mitochondrial damages. *Saudi Journal of Biological Sciences*, *27*(11), pp.3018-3024.

[4] Illian, D.N., Basyuni, M., Wati, R. and Hasibuan, P.A.Z., 2018. Polyisoprenoids from Avicennia marina and Avicennia lanata inhibit WiDr cells proliferation. *Pharmacognosy Magazine*, *14*(58), p.513.

[5] Eswaraiah, G., Peele, K.A., Krupanidhi, S., Kumar, R.B. and Venkateswarulu, T.C., 2020. Identification of bioactive compounds in leaf extract of Avicennia alba by GC-MS analysis and evaluation of its in-vitro anticancer potential against MCF7 and HeLa cell lines. *Journal of King Saud University-Science*, *32*(1), pp.740-744.

Chapter 4

Materials and Methods

4. Materials and Methods:

4.1. Collection of sample & its Preparation

Fresh *A. marina* leaves were collected from mangrove forest in Sunderbans, West Bengal. For the extraction of bioactive compounds, whole leaves were blended using a common household mixer-grinder machine and after that, the freshly produced powdered leaf extract was kept in 40% ethanol overnight in refrigerator. Next day it was filtered under vacuum pressure using Whatman Filter paper no. 1. Filtered extract solution was concentrated using rotary evaporator (manufacturer: IKA). Then the concentrated extract solution was lyophilized using lyophilizer (manufacturer: Biobas) for 24 hrs. at -70º C. Lyophilized leaf powder was obtained as sample for future testing. The whole powdered product was contained within an Eppendorf and stored at -3º C.

4.2. Phytochemical Screening Tests for the Leaf Extract

All the phytochemical tests were performed following standard protocol. Crude leaf extract was manufactured from whole *A. marina* leaf. This extract was screened through several tests for the identification of Phenols, Flavonoids, Tannin, Alkaloids, and Reducing sugars present in the plant leaf.

4.2.1. Test for Phenols

Ferric Chloride test: 2 mL of leaf extract was taken in a test tube. Then very few drops of 5% ferric chloride solution were mixed with it. The resultant solution appeared green-blue in color. This change in color of the leaf extract solution in turn confirmed the presence of phenols in the plant leaf under study. [1]

4.2.2. Test for flavonoids

Ammonia test: About 2 mL of aqueous filtrate of leaf extract was taken in a test tube. Then 3 mL of dilute ammonia was added to the leaf extract. 1 mL concentrated sulphuric acid (H_2SO_4) was then added to the test tube. A yellow color appeared, which proved that flavonoids are indeed present in the plant leaves. [1]

4.2.3. Test for reducing sugars

Fehling's test: 0.2 g of powdered leaf sample was added in 1 mL ethanol. The solution was then added to 3 mL of distilled water and mixed. 1 ml of Fehling's solution A and B was taken

in a test tube and heated to boiling point and then poured in the aqueous ethanolic leaf extract. Change in color (brick red precipitation) confirmed the presence of reducing sugars. [2]

4.2.4. Test for alkaloids

0.1 gm powdered leaf sample was weighed using a weight machine. Then it was carefully poured in a clean glass test tube. 2 mL of hexane was added to the sample. Then the test tube was shaken well for sometimes. Filtration was performed immediately after that. Now, 3 mL of 2% HCL acid was added to the aforementioned filtrate. The new solution was then heated and thoroughly filtered. One drop of picric acid was added to the filtrate which in turn developed yellow precipitate indicating the presence of alkaloids. [3]

4.2.5. Test for Tannins

At first 2mL of the leaf extract was diluted with water. 3 - 4 drops of 10% ferric chloride solution was then poured carefully inside the glass test tube containing the diluted extract. The color of the solution changes to blue-green as gallic tannins and catecholic tannins were present respectively in the leaf extract. [3]

4.2.6. Test for Saponins

Foam test - 1ml leaf extract was added to 20 mL of distilled water in a measuring cylinder and then it was shaken for 15 minutes. Development of stable foam determined the presence of saponins. [4]

4.3. Evaluation of Total Phenolic content (TPC)

Materials required: Folin-Ciocalteau reagent (FCR), Gallic acid, Sodium carbonate, distilled water

Instrument required: UV-vis Spectrophotometer (Agilent Technologies, Cary 60)

With the use of Folin-ciocalteau (FC) reagent, total phenolic content of *A. marina* leaf extract was determined. 0.5 ml of A. marina leaf extract was added to 0.5 mL of FC reagent (2x diluttion with water). The new solution was allowed to incubate up to 5 minutes at 25° C; this step was followed by the addition of 2 mL of 20% Na_2Co_3. The mixture was further incubated at 25° C for 90 minutes. Using a UV-Vis spectrophotometer the absorbance of the solution got measured at 650 nm. Using the OD values for different concentrations of Gallic acid, a standard curve of Gallic acid was prepared. Then the total phenolic content (µg/ml) was calculated using Gallic acid as reference. [5, 6]

4.4. Evaluation of Total Flavonoid content (TFC)

Materials required: Sodium nitrite ($NaNO_2$), Aluminium chloride ($AlCl_3$), Sodium hydroxide (NaOH), Quercetin ($C_{15}H_{10}O_7$)

Instrument required: UV-visible Spectrophotometer (Agilent Technologies, Cary 60)

A standard curve of quercetin was prepared. TFC of extracted sample was calculated from that standard curve with the help of spectrophotometric analysis. Here, 0.5 mL of each different concentration of plant extract (0 to 500 µg/ml) and quercetin (of different concentrations from 0-500 µg/mL) were taken in the respective test tubes. Then 0.5 mL of distilled water was added to the test tubes. After that, 0.3 mL of 5% $NaNO_2$ was added to it and kept at room temperature (25°C) for 5 minutes. Then 0.3 mL of 10 % aqueous solution of $AlCl3_3$ was added. After 5 minutes, 2 mL of 1 molar NaOH was added to the reaction solution and all the test tubes were shaken vigorously before taking the absorbance at 510 nm against a blank (only water without any sample solution) using UV-visible Spectrophotometer. All results were determined using a standard curve of quercetin and a linear equation was used to estimate the total amount of flavonoids present in the plant extract. [7]

4.5. Antibacterial assay

Materials required: Nutrient broth, Agar agar, streptomycin, Distilled water

Test Microorganisms: Standard strains of bacteria, *Escherichia coli* (gram negative) and *Bacillus subtilis* (gram positive)

To check the potency of *A. marina* leaf extracts against the above mentioned two bacterial strains, 50 µL of crude sample (leaf extract) was used in this test which was carried out using the well-diffusion technique to determine the antibacterial activity against *E. coli* and *B. subtilis*. Water was taken as the control and streptomycin acted as the standard. Plates were incubated for 24h and the zone of inhibition was observed. [9]

4.6. Antioxidant activity - by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

Materials required: DPPH ($C_{18}H_{12}N_5O_6$), Ascorbic Acid ($C_6H_8O_6$)

Instrument required: UV-visible Spectrophotometer (Agilent Technologies, Cary 60)

Free radical scavenging activity (RSA) was performed with the use of DPPH method. Different concentrations of the A. marina leaf extracts (25, 50, 75, 100 µg/mL) were used. 200 µL of

0.02% DPPH solution was added to 50µL of all concentrations of extracts separately in a dark room. The micro well plate was then carefully and securely wrapped with aluminium foil and incubated for 30 min at room temperature in dark. The absorbance was taken at 595 nm in triplicates for each concentration. We took Ascorbic acid as a standard antioxidant. Water was used as control. The percentage inhibition of free radical formation was calculated according the formula below:

$$\boldsymbol{RSA}\ (\%) = \frac{\boldsymbol{OD}(\mathbf{Control}) - \mathbf{OD}(\mathbf{Sample})}{\mathbf{OD}(\mathbf{Control})}\ \mathbf{X\ 100}$$

Here RSA (%) is the Percentage of Radical Scavenging Activity which is in other words the percentage of DPPH scavenged. All the tests were performed in triplicates. [6, 7]

4.7. Anti-inflammatory activities – by Bovine Serum Albumin Assay (BSA)

The anti-inflammatory activities of the test plant extract of different concentrations were measured using a modified version of the BSA assay reported by Williams et al. 0.4% BSA solution was prepared in Tris Buffered Saline (one tablet is dissolved in 15 mL of deionized water to yield 0.05M Tris and 0.15M sodium chloride, pH 7.6 at 25 ^{0}C). The pH was set to 6.4 with the addition of glacial acetic acid accordingly. From stock solution (100mg/mL) five concentrations of plant extract was prepared - 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL and 250 µg/mL. All these sample solution with different concentrations were then added to separate Eppendorf tubes containing 200 µL of 0.4%, w/v BSA buffer solution. We took methanol as negative control and aspirin as positive control; both were assayed in a same way described earlier. The solutions were then heated in a water bath at 72 °C for 10 minutes, and cooled for 20 minutes under laboratory conditions. The turbidity of the solutions (level of protein precipitation) was measured at 660 nm using the Spectrophotometer. The experiments were conducted in duplicates and the mean OD values were noted. The percentage inhibition of precipitation (i.e. anti - protein denaturation) was done relative to the negative control, based on the following equation:

$$\%\ \boldsymbol{Anti - Denaturation\ Activity} = \frac{\boldsymbol{OD}\ (\boldsymbol{Control}) - \boldsymbol{OD}\ (\boldsymbol{Sample})}{\mathbf{OD}\ (\boldsymbol{Control})}\ \mathbf{X\ 100}$$

% Anti-Denaturation Activity = % Inhibition of Protein Denaturation = % Anti-inflammatory Activity [8]

4.8. Cytotoxic test – MTT assay

Materials required: DMEM (Dulbecco's Modified Eagle Medium**)**, FBS (Foetal Bovine Serum), penicillin-streptomycin, MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), PBS (Phosphate buffered saline), DMSO (Dimethyl sulfoxide)

Mouse Fibroblast cells (L929 cell line) were cultured in DMEM. Cells were added in 96 well plates and cultured at 37 ^{0}C for 24 h in a humidified incubator with 5% CO_2. After 24 h, L929 fibroblast cells went under treatment with various concentrations of A. marina leaf extracts (25, 50, 100, 250, 500 µg/mL) as well as standard drug penicillin-streptomycin. Then the plates were incubated at 37 ^{0}C in 5% CO_2 for 24 h. After that, 50µL of MTT (concentration 1mg/ml in PBS) was poured drop by drop carefully using a pipette inside each well and further incubated for 3-4 hrs. Then MTT along with the media was pipetted out very carefully and 100 µL of DMSO was added per well to dissolve the newly formed formazan crystals. The absorbance was taken at 570 nm using the UV-vis spectrophotometer. Percentage viability of the cells was determined using the below formula- [9] [10]

$$\%\ \boldsymbol{Viable\ Cell} = \frac{\boldsymbol{Absorbance\ of\ treated\ cells}}{\boldsymbol{Absorbance\ of\ untreated\ cells}}\ \mathbf{X\ 100}$$

4.9. Hemocompatibility test

This was performed based on ASTM (American Society for Testing and Materials) standard. It determined the extent of haemolysis at the presence of the sample in blood. Blood was collected in EDTA vials (Ethylenediamine tetraacetic acid**)** to avoid clotting. Dilution of blood was then done using normal saline at a proportion of 4:5. To check haemolysis, 0.2 mL diluted blood was mixed with 9.8 mL of distilled water and incubated at 37 ^{0}C for 60 minutes after which the OD (Optical Density) was taken in a spectrophotometer at 545 nm. In general when distilled water comes in contact of blood, it causes large-scale rupture of RBCs (Red Blood Corpuscles) so the OD value of Distilled H_2O was considered as positive control [OD (+)]. For the negative control, further dilution of 0.2 mL diluted blood was done using 10 mL of normal saline solution. Now it was incubated at 37 ^{0}C for 60 min. As normal saline solution causes least rupture of RBCs so it was taken as negative control. The OD value of this solution was measured in a spectrophotometer at 545 nm and was marked as OD (-). Similar procedure was followed with all the test samples and their respective OD values were noted. The samples were taken in falcon tubes containing 9.8 mL normal saline, then put to incubation at the temperature of 37 ^{0}C, for a time period of 30 minutes in order to reach temperature equilibrium.

0.2 mL of diluted blood was then added to the tubes, gently mixed and again put to incubation for another 60 minutes. Finally, solutions were centrifuged at 2000 rpm for 5 minutes and the OD (sample) values were recorded. [9]

$$\%\ \boldsymbol{Hemolysis} = \frac{[\boldsymbol{OD}\ (\boldsymbol{sample}) - \boldsymbol{OD}\ (-)]}{[\boldsymbol{OD}\ (+) - \ \boldsymbol{OD}\ (-)]}\ \mathbf{X\ 100}$$

4.10. In-silico Study:

4.10.1. Phytochemicals found in A. marina leaf

Going through different papers (provided by the google scholar section where different published scientific papers are available for studying purposes) written on phytochemicals ('phyto' means plant & 'phytochemical' means the chemical compounds exclusively produced by the plants) found in A. marina mangrove plant, the name of the compounds were noted in a table. After noting down all the available phytochemical found throughout the whole parts of the plant (like fruit, flower, shoot, bark, leaf and so on), only the phytochemicals that are exclusively produced in the plant's leaves, were selected. It has been done this way because for our current study, we want to concentrate on the A. marina leaves only.

After the phytochemicals found in A. marina leaf were selected, we took help from another website i.e. ***'PubChem'*** website which is a treasury for the details of any chemical compound that has been discovered till today. To say in details, PubChem is a type of database for chemical molecules. This website acts as a reservoir of vast information regarding every compound that has been discovered till today. It also stores all the different types of activity of the molecules against several biological assays. The whole system is governed and monitored by the National Centre for Biotechnology Information (NCBI). It is a part of the National Library of Medicine (NLM), which itself is a component of the United States National Institutes of Health (NIH). The above mentioned databank is a free public access portal that can be accessed through a web user interface. Almost all the molecular structures (3D & 2D) and detailed descriptive datasets for each compound can also be downloaded without any cost with the help of file transfer protocol (FTP). More than 80 database vendors regularly add their updated information to the ever-growing PubChem databank. So, with the help of this vast database based website, the details of our chemicals of interest were downloaded and put in the Table 4.1 below.

In the PubChem website the name of each compound was put in the search box and the respective details were collected for each of the molecules. The details like- ***Canonical SMILES***, molecular weight and three dimensional (3D) structures of all the chemicals under study, have been gathered from the above-mentioned website. The whole data set is provided is the table 4.1 below. SMILES is the abbreviation of 'Simplified Molecular Input Line Entry System'. It is one type of chemical notation of the chemical compound. This unique notation allows the user to represent a chemical structure in a way that can be used/ read by the computer. The canonical form of the SMILES is a linear text format. This particular orderly arrangement of meaningful texts can describe the connectivity and chirality of a molecule of interest. There is another form of SMILES called Isomeric SMILES which can come into use in case of for some molecules whose Canonical SMILES are unavailable or yet to be computed out by the researchers. Then we can use the isomeric SMILES instead of the Canonical SMILES for the next step of our research work. In the table 4.1 below for the first 35 compounds, we have put their Canonical SMILES but in case of 36th to 42nd compounds as the Canonical SMILES were unavailable, we have put the Isomeric SMILES in use.

Table- 4.1. Details of the A. marina leaf phytochemicals using PubChem [11]

Sl. No.	Chemical Compound	Canonical / Isomeric SMILES	Mol. Wt.
1	Squalene	CC(=CCCC(=CCCC(=CCCC=C(C)CCC=C(C)CCC=C(C)C)C)C)C	410.7
2	2(R),3(S)-1,2,3,4-Butane tetrol	C(C(C(CO)O)O)O	122.12
3	n-Hexadecanoic acid	CCCCCCCCCCCCCCCC(=O)O	256.42
4	Hexadecanoic acid, ethyl ester	CCCCCCCCCCCCCCCC(=O)OCC	284.5
5	3,7,11,15-tetramethyl-2-hexadecan-1-ol	CC(C)CCCC(C)CCCC(C)CCCC(=CCO)C	296.5
6	Phytol	CC(C)CCCC(C)CCCC(C)CCCC(=CCO)C	296.5
7	(E)-9-Octadecenoic acid ethyl ester	CCCCCCCCC=CCCCCCCCC(=O)OCC	310.5
8	Dodecanoic acid/lauric acid	CCCCCCCCCCCC(=O)O	200.32
9	Octadecanoic acid,2-methyl-,methyl ester	CCCCCCCCCCCCCCCCCC(C)C(=O)OC	312.5
10	cis-9-Hexdecenal	CCCCC=CCCCCCCCCC=O	238.41
11	D-Allose	C(C1C(C(C(C(O1)O)O)O)O)O	180.16
12	Nonanoic acid	CCCCCCCCC(=O)O	158.24
13	Isorhamnetin-3-O-rutinoside	CC1C(C(C(C(O1)OCC2C(C(C(C(O2)OC3=C(OC4=CC(=CC(=C4C3=O)O)O)C5=CC(=C(C=C5)O)OC)O)O)O)O)O)O	624.5
14	Quercetin	C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O)O	302.23
15	Kaempferol	C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O	286.24
16	Lupeol	CC(=C)C1CCC2(C1C3CCC4C5(CCC(C(C5CCC4(C3(CC2)C)C)(C)C)O)C)C	426.7
17	Betulin	CC(=C)C1CCC2(C1C3CCC4C5(CCC(C(C5CCC4(C3(CC2)C)C)(C)C)O)C)CO	442.7
18	α-amyrin	CC1CCC2(CCC3(C(=CCC4C3(CCC5C4(CCC(C5(C)C)O)C)C)C2C1C)C)C	426.7
19	Linolenic acid	CCC=CCC=CCC=CCCCCCCCC(=O)O	278.4
20	Stearic acid	CCCCCCCCCCCCCCCCCC(=O)O	284.5

Sl. No.	Chemical Compound	Canonical / Isomeric SMILES	Mol. Wt.
21	Myristic acid	CCCCCCCCCCCCCC(=O)O	228.37
22	Geniposidic acid	C1C=C(C2C1C(=COC2OC3C(C(C(C(O3)CO)O)O)O)C(=O)O)CO	374.34
23	Mussaenoside	CC1(CCC2C1C(OC=C2C(=O)OC)OC3C(C(C(C(O3)CO)O)O)O)O	390.4
24	Verbascoside	CC1C(C(C(C(O1)OC2C(C(OC(C2OC(=O)C=CC3=CC(=C(C=C3)O)O)CO)OCCC4=CC(=C(C=C4)O)O)O)O)O)O	624.6
25	Isoverbascoside	CC1C(C(C(C(O1)OC2C(C(OC(C2O)OCCC3=CC(=C(C=C3)O)O)COC(=O)C=CC4=CC(=C(C=C4)O)O)O)O)O)O	624.6
26	Syringaresinol	COC1=CC(=CC(=C1O)OC)C2C3COC(C3CO2)C4=CC(=C(C(=C4)OC)O)OC	418.4
27	Chrysoeriol 7-Oglucoside	COC1=C(C=CC(=C1)C2=CC(=O)C3=C(C=C(C=C3O2)OC4C(C(C(C(O4)CO)O)O)O)O)O	462.4
28	5-hydroxy-4, 7-dimethoxyflavone	COC1C(=O)C(OC2=CC(=CC(=C12)O)OC)C3=CC=CC=C3	300.3
29	4',5'-Dihydroxy-3,7,3'-trimethoxyflavone	COC1=CC2=C(C=C1)C(=O)C(=C(O2)C3=CC(=C(C(=C3)OC)O)O)OC	344.3
30	4',5-Dihydroxy-3',5'-dimethoxyflavone	COC1=CC(=CC(=C1O)OC)C2=CC(=O)C3=C(C=CC=C3O2)O	314.29
31	3',4',5-trihydroxy-7-methoxyflavone	COC1=CC(=C2C(=C1)OC(=C(C2=O)O)C3=CC=C(C=C3)O)O	300.26
32	ß-sitosterol	CCC(CCC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C)C(C)C	414.7
33	Ergost-6,22-diene-5,8-epidioxy-3ß-ol	CC(C)C(C)C=CC(C)C1CCC2C1(CCC3C24C=CC5(C3(CCC(C5)O)C)OO4)C	428.6
34	Palmitic acid	CCCCCCCCCCCCCCCC(=O)O	256.42
35	2'-Cinnamoylmussae-nosidic acid	CC1(CCC2C1C(OC=C2C(=O)O)OC3C(C(C(C(O3)CO)O)O)OC(=O)C=CC4=CC=CC=C4)O	506.5
36	7-O-5-phenyl-2,4-pentadienoyl-8-epiloganin	CC1(C(CC2C1C(OC=C2C(=O)OC)OC3C([C@@H]([C@@H](C(O3)CO)O)O)O)COC(=O)/C=C/C=C/c4ccccc4)C	574.623
37	10-O-[(E)-p-cinna-moyl]-geniposidic acid	c1ccc(cc1)/C=C/C(=O)OCC2=CCC3C2C(OC=C3C(=O)O)O[C@H]4[C@H]([C@@H](C([C@H](O4)CO)O)O)O	504.488
38	Marinoid A	CC1(CCC2C1C(OC=C2C(=O)O)OC3[C@@H]([C@@H](C([C@H](O3)CO)O)O)OC(=O)/C=C/c4ccccc4)O	506.504
39	Marinoid B	CC1(CCC2C1C(OC=C2C(=O)O)OC3[C@@H]([C@@H](C([C@H](O3)CO)O)O)OC(=O)/C=C/c4ccc(cc4)OC)O	536.53
40	Marinoid C	CC1(CCC2C1C(OC=C2C(=O)O)OC3[C@@H]([C@@H](C([C@H](O3)CO)O)O)OC(=O)/C=C/c4ccc(cc4)O)O	522.503
41	Marinoid D	COc1cc(cc(c1O)OC)C(=O)OCC2=CCC3C2C(OC=C3C(=O)O)OC4[C@@H]([C@@H](C([C@H](O4)CO)O)O)O	554.501
42	Marinoid E	c1ccc(cc1)/C=C/C(CC(=O)OCC2=CCC3C2C(OC=C3C(=O)O)OC4[C@@H]([C@@H](C([C@H](O4)CO)O)O)O)O	548.541
43	p-methoxy cinnamic acid	COC1=CC=C(C=C1)C=CC(=O)O	178.187

4.10.2. Screening of phytochemicals through ADMET property checking & Selection of phytochemicals for docking [12]

ADMEtlab2.0 is a freely available, user-friendly website for checking ADMET properties of chemical compounds based on a huge and comprehensive database.

Here the canonical SMILES of all the above mentioned phytochemicals were put and based on their individual properties, some were selected for docking purpose and others got rejected.

The comparative table prepared below is based on the following properties-

- *Molecular Weight (MW):* It is a very important physiochemical property which is the first criterion for selection of a molecule as a candidate for potential drug. Optimal MW: 100~600, based on Drug-Like Soft rule.
- *Caco-2 Permeability:* The human colon adenocarcinoma cell permeability is an important property for absorption.

  Any oral drug before reaching the systemic circulation should pass through intestinal cell membranes with the help of passive diffusion, carrier-mediated uptake or active transport system. Caco-2 is used in vivo as an alternative approach for the human intestinal epithelium, for permeability testing of drugs due to their morphological and functional similarities with each other. Therefore it is clear that Caco-2 cell permeability is obviously a very important parameter for all the potential drug candidates.

  Interpretation of Results: The result is given as the log cm/s. If the compound's predicted value >-5.15log cm/s then the compound has a proper Caco-2 permeability.

  Empirical decision: > -5.15: excellent (green); otherwise: poor (red)

- *HIA (Human intestinal absorption):* This is another necessary parameter to check whether a potential drug can achieve the status of being an effective oral drug or not. So, human intestinal absorption of a drug which is taken orally, is primarily essential for checking the drug's efficacy to be considered when taking it orally.

  There is a proved close relationship between oral bioavailability and intestinal absorption. Therefore HIA could be considered as an alternate for oral bioavailability parameter.

  Interpretation of Result: Compounds having less than 30% absorbance generally gets absorbed poorly. According to the established rule, molecules whose HIA >30% fall in the HIA- (Category 0); molecules whose HIA < 30% belong to HIA+ (Category 1). The output value is the probability of being HIA+, with a range of 0 to 1.

  Empirical decision: 0-0.3: excellent (green); 0.3-0.7: medium (yellow); 0.7-1.0(++): poor (red)

- *PPB:* Plasma protein binding is considered as one of the prime mechanisms for uptake and distribution of drugs.

So, when a drug gets bound with plasma protein, its pharmacodynamics behaviour gets strongly influenced by that particular incidence.
PPB has direct effect on oral bioavailability as free concentration of a particular drug gets compromised when a drug gets attached to serum or plasma proteins.

Interpretation of Result: If a compound's predicted value < 90% then it is said to have a proper PPB; drugs that have high affinity to get bound with the protein, may have a low therapeutic index.

Empirical decision: ≤ 90%: excellent (green); otherwise: poor (red).

- *CL (Clearance of a drug):* This is another important parameter to consider for checking on a potential drug candidate. It is a pharmacokinetic indicator.
  With addition to the volume of distribution this parameter defines the half-life of a drug; so basically it gives us an idea about the frequency for the dose of a drug.

  Interpretation of Result: The unit is mL/min/kg. >15 mL/min/kg indicates high clearance; 5- 15 mL/min/kg indicates moderate clearance; < 5 mL/min/kg indicates low clearance

  Empirical decision: ≥ 5: excellent (green) ;< 5: poor (red).

- *PAINS:* The full form is Pan Assay Interference Compounds. It is one among many famous frequent hitter filters. This filter is composed of 480 substructures derived from the FHs analysis and FHs were determined by six target-based HTS assays.
  When this filter is applied, it is easier to screen for false positive hits. It can also flag suspicious and unsuitable compounds from the database that is getting screened.
  One of the most famous medicinal magazines, Journal of Medicinal Chemistry have set some pre-established requirements for authors so that they must provide their screening results along with PAINS alerts of active compounds when submitting their articles.
- *Lipinski Rule:* Conditions → MW≤500 DA;
  logP≤5;
  Hacc≤10;
  Hdon≤5

  Abiding this rule is considered most important as that means good in vivo drug absorption and permeation.

Poor absorption and permeation generally occur when –

I) the number of H-bond donors exceeds 5,
II) the number of H-bond acceptors exceeds 10,
III) MW is greater than 500 DA, and
IV) log P value is greater than 5.

LogP: The logarithm of the n-octanol/water distribution coefficient.

Log P has sufficient impact on membrane permeability and hydrophobic binding to macromolecules; these macromolecules can be the target receptors, other proteins e.g. plasma proteins, transporters, metabolic enzymes etc.

The predicted value of logP for a compound is presented in the form of the logarithm of its molar concentration (log mol/L).

Hacc: Hydrogen bond acceptors. Expressed as the sum of Ns and Os.

Hdon: Hydrogen bond donors; that is the sum of OHs and NHs.

Interpretation of Result: If two conditions, as described above, gets out of range, very poor absorption/ permeability is seen; deviation of a single property is considered acceptable.

Empirical decision: < 2 violation is considered excellent (green); ≥2 violations are considered as poor (red)

- *Pfizer Rule:* Content: logP > 3;
  TPSA < 75

Interpretation of Result: Following this rule, it can be concluded that compounds having high log P value, which is >3 and low TPSA i.e. <75 are most definitely going to be toxic in nature. So those compounds are not at all suitable as potential drugs for any diseases. In the opposite case the molecules can be considered for further drug development studies.

Empirical decision: when above two conditions are satisfied the result is considered poor (red); otherwise, excellent (green) [12]

Table 4.2. Comparative table for A. marina leaf phytochemicals based on ADMET properties

[12]

Sl. No	Phytochemicals	MW	Caco-2	HIA	PPB	CL	PAINS	Lipinski	Pfizer
1	Squalene	410.39	-4.705	0.005	86.48%	13.462	0	Accepted	Rejected
2	2(R),3(S)-1,2,3,4-Butane tetrol	122.06	-5.137	0.813	9.78%	2.225	0	Accepted	Accepted
3	n-Hexadecanoic acid	256.24	-5.027	0.005	98.95%	2.377	0	Accepted	Rejected
4	Hexadecanoic acid, ethyl ester	284.27	-4.764	0.001	97.49%	4.647	0	Accepted	Rejected
5	3,7,11,15-tetramethyl-2-hexadecan-1-ol	296.31	-4.338	0.002	97.64%	8.161	0	Accepted	Rejected
6	Phytol	296.31	-4.338	0.002	97.64%	8.161	0	Accepted	Rejected
7	(E)-9-Octadecenoic acid ethyl ester	310.29	-4.69	0.002	97.54%	5.38	0	Accepted	Rejected
8	Dodecanoic acid/lauric acid	200.18	-4.898	0.005	96.81%	2.259	0	Accepted	Rejected
9	Octadecanoic acid,2-methyl-,methyl ester	312.3	-4.857	0.002	97.42%	4.788	0	Accepted	Rejected
10	cis-9-Hexdecenal	238.23	-4.669	0.005	93.25%	4.379	0	Accepted	Rejected
11	D-Allose	180.06	-5.318	0.899	12.50%	1.474	0	Accepted	Accepted
12	Nonanoic acid	158.13	-4.823	0.005	92.67%	3.242	0	Accepted	Rejected
13	Isorhamnetin-3-O-rutinoside	624.17	-6.298	0.927	79.23%	1.375	0	Rejected	Accepted
14	Quercetin	302.04	-5.204	0.014	95.50%	8.284	0	Accepted	Accepted
15	Kaempferol	286.05	-4.974	0.008	97.86%	6.868	0	Accepted	Accepted
16	Lupeol	426.39	-5.02	0.008	98.80%	17.929	0	Accepted	Rejected
17	Betulin	442.38	-4.954	0.009	97.22%	15.799	0	Accepted	Rejected
18	α-amyrin	426.39	-4.953	0.008	99.70%	17.973	0	Accepted	Rejected
19	Linolenic acid	278.22	-4.631	0.007	97.12%	4.877	0	Accepted	Rejected
20	Stearic acid	284.27	-5.068	0.005	99.22%	2.425	0	Accepted	Rejected
21	Myristic acid	228.21	-4.96	0.005	98.20%	2.303	0	Accepted	Rejected
22	Geniposidic acid	374.12	-6.006	0.93	23.05%	1.598	0	Accepted	Accepted
23	Mussaenoside	390.15	-5.7	0.93	23.26%	1.617	0	Accepted	Accepted
24	Verbascoside	624.21	-6.449	0.993	93.34%	1.702	1	Rejected	Accepted
25	Isoverbascoside	624.21	-6.418	0.99	95.17%	1.727	1	Rejected	Accepted
26	Syringaresinol	418.16	-4.784	0.014	82.05%	6.418	0	Accepted	Accepted
27	Chrysoeriol 7-Oglucoside	462.12	-5.976	0.856	80.28%	4.068	0	Rejected	Accepted
28	5-hydroxy-4, 7-dimethoxyflavone	300.1	-4.722	0.006	94.77%	6.931	0	Accepted	Accepted
29	4',5'-Dihydroxy-3,7,3'-trimethoxyflavone	344.09	-4.839	0.015	88.61%	5.403	1	Accepted	Accepted
30	4',5-Dihydroxy-3',5'-dimethoxyflavone	314.08	-4.871	0.032	91.35%	4.406	0	Accepted	Accepted
31	3',4',5-trihydroxy-7-methoxyflavone	300.06	-4.892	0.009	97.71%	5.318	0	Accepted	Accepted
32	ß-sitosterol	414.39	-4.756	0.004	98.31%	16.686	0	Accepted	Rejected
33	Ergost-6,22-diene-5,8-epidioxy-3ß-ol	428.33	-4.756	0.006	98.86%	15.865	0	Accepted	Rejected
34	Palmitic acid	256.24	-5.027	0.005	98.95%	2.377	0	Accepted	Rejected
35	2'-Cinnamoylmussaenosidic acid	506.18	-6.094	0.978	74.54%	1.387	0	Rejected	Accepted
36	7-O-5-phenyl-2,4-pentadienoyl-8-epiloganin	574.24	-5.264	0.787	78.26%	1.534	0	Rejected	Accepted
37	10-O-[(E)-p-cinnamoyl]-geniposidic acid	504.16	-5.882	0.887	71.58%	1.464	0	Rejected	Accepted
38	Marinoid A	506.18	-6.043	0.926	69.58%	1.466	0	Rejected	Accepted
39	Marinoid B	536.19	-6.022	0.939	71.56%	1.413	0	Rejected	Accepted
40	Marinoid C	522.17	-6.274	0.975	78.15%	1.457	0	Rejected	Accepted
41	Marinoid D	554.16	-6.395	0.939	55.95%	2.54	0	Rejected	Accepted
42	Marinoid E	548.19	-6.023	0.943	60.86%	1.684	0	Rejected	Accepted
43	p-methoxy cinnamic acid	178.06	-4.7	0.011	89.42%	4.293	0	Accepted	Accepted

From the above comparative table of *A. marina* leaf phytochemicals, we could see that 13 out of the total 43 leaf phytochemicals got rejected after ADMET screening was done.

Most of their property scores didn't match with the accepted range of score values; this was the reason behind them getting rejected for further studies. [12]

So (43-13) = 30 leaf phytochemicals were seen to get the scores in the range of accepted values. Only those 30 phytochemicals were selected for the next step of the study.

These 30 compounds are now going to get docked acting as the ligands with human breast cancer protein HER2 acting as the receptor.

4.10.3. Receptor and ligand preparation

The 3D structure of HER2 breast cancer protein (receptor) was downloaded from ***RCSB PDB*** (protein data bank) website [15]. Phytochemical 3D structures were downloaded as SDF file format from ***PubChem*** website [11]. With the help of ***BIOVIA Discovery Studio 2021*** software [13] the SDF files were converted to PDB format.

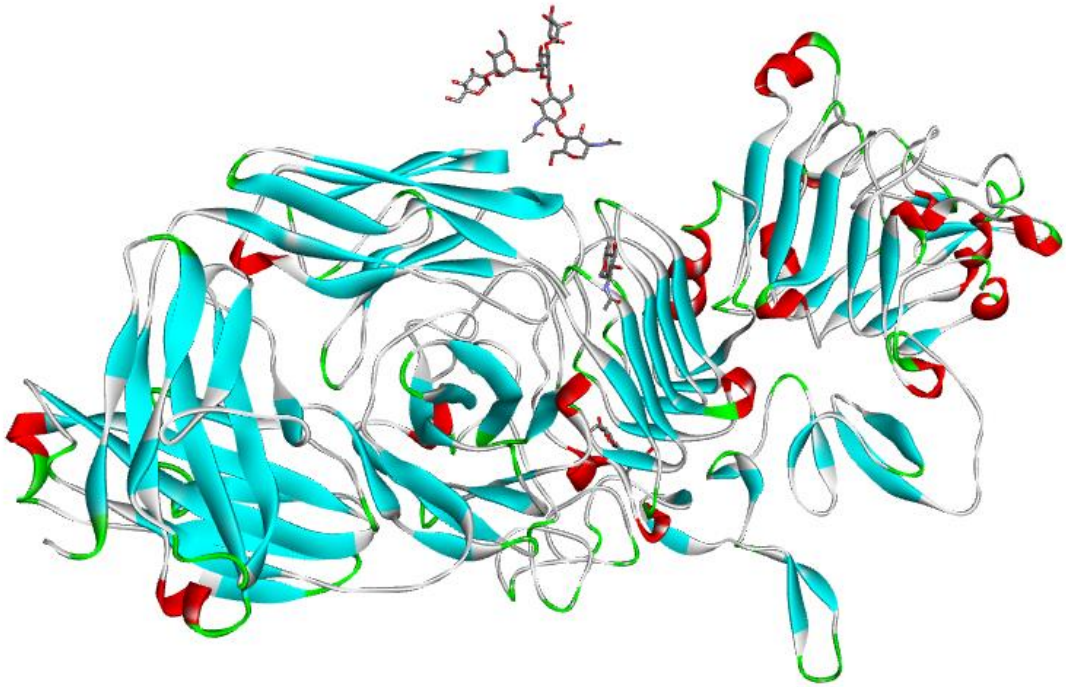

Fig.4.1. The 3D structure of HER2 in complex with pre-existing Fab MF3958 (PDB ID: 5O4G)

Preparation of receptor prior docking: The HER2 protein file was opened in Discovery Studio. Then the pre-attached ligand groups are deleted from the whole structure. Now the new structure containing only the HER2 protein groups (namely A, B, C chains) only was saved as PDB file by replacing the existing file.

Preparation of ligand prior docking: The phytochemical (SDF file format) 3D structures downloaded from PubChem were opened in Discovery Studio and saved as PDB files.

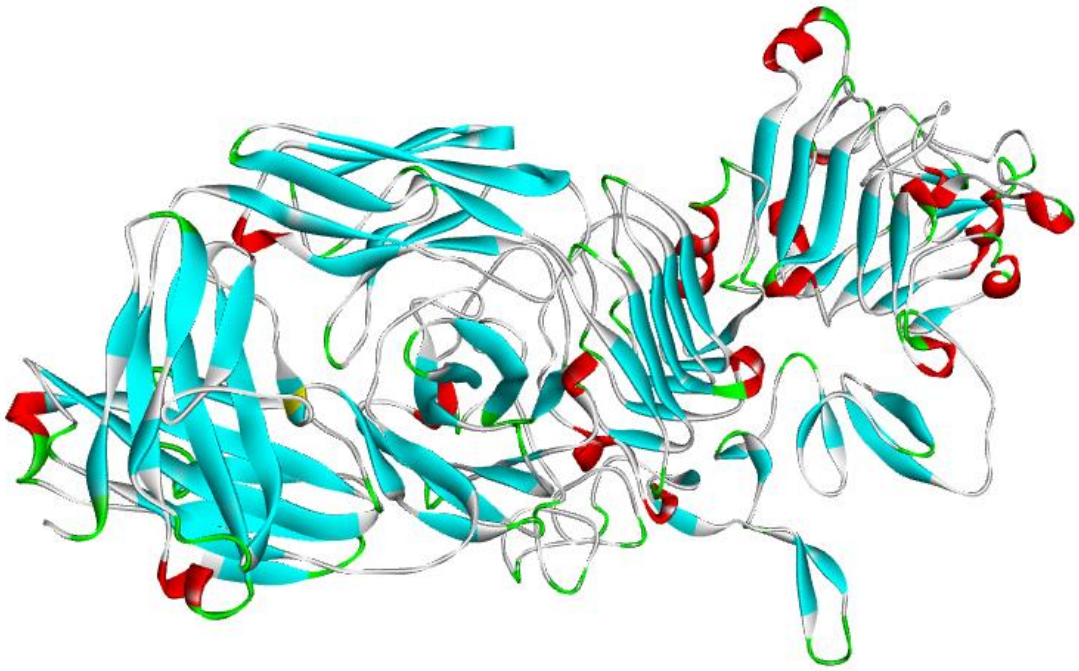

Fig.4.2. 3D Structure of HER2 protein after deletion of pre-existing ligand

After that, ***Autodock tools – 1.5.7*** (Autodock Vina) [14] is opened. Now the protein PDB file was read. Some edits were done like if water molecules existed then those were deleted then only polar hydrogens were added, Kollman charges were added. In brief, hydrogens were added to the system in order to optimize the ligand and the receptor. Then specific charges were assigned, for associating the atoms with partial charges.

4.10.4. Active site selection

The active site of the Breast Cancer HER2 Protein (PDB ID: 5O4G) was selected from different receptor-cavities available in the molecular structure of the compound. The site having specific coordinates (X= 55.9, $Y = 87.7$, $Z = 78.5$) was selected as the active site because the native inhibitor ligand and standard drugs for HER2+ breast cancer (Neratinib, Lapatinib) have affinity for the particular site.

4.10.5. Virtual molecular docking

Molecular docking and analysis part was done virtually based on the method described previously by Majumder et al., 2019. All the ligands were docked through Autodock Vina to active site of HER2 protein, having coordinates of X= 55.9, $Y = 87.7$, $Z = 78.5$. Re-docking was also performed to validate the virtual molecular docking protocol. [16]

References:

1. Vimalkumar CS, Hosagaudar VB, Suja SR, Vilash V, Krishnakumar NM, Latha PG. Comparative preliminary phytochemical analysis of ethanolic extracts of leaves of Olea dioica Roxb., infected with the rust fungus Zaghouania oleae (E.J. Butler) Cummins and non-infected plants. J Pharmacogn Phytochem 2014;3(4):69-72.

2. Thilagavathi, T. & Rajasekar, Arvindganth & Doss, Vidhya & Ravichandran, Dhivya. (2015). PRELIMINARY PHYTOCHEMICAL SCREENING OF DIFFERENT SOLVENT MEDIATED MEDICINAL PLANT EXTRACTS EVALUATED. International Research Journal of Pharmacy. 6. 246-248. 10.7897/2230-8407.06455.

3. Basak, Piyali & Paul, Samrat & Majumder, Ranabir. (2017). Invitro cytotoxic study of aloe vera whole leaf extract on PBMC and breast cancer cell line. 124-127. 10.1109/I2CT.2017.8226106.

4. Shaikh, Junaid & Patil, Matsyagandha. (2020). Qualitative tests for preliminary phytochemical screening: An overview. 8. 603-608. 10.22271/chemi.2020.v8.i2i.8834.

5. Vernon L. Singleton, Rudolf Orthofer, Rosa M. Lamuela-Raventós, [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent, Methods in Enzymology, Academic Press, Volume 299, 1999, Pages 152-178, ISSN 0076-6879, ISBN 9780121822002, https://doi.org/10.1016/S0076-6879(99)99017-1.
(https://www.sciencedirect.com/science/article/pii/S0076687999990171)

6. Hafiza Noreen, Nabil Semmar, Muhammad Farman, James S.O. McCullagh, Measurement of total phenolic content and antioxidant activity of aerial parts of medicinal plant Coronopus didymus, Asian Pacific Journal of Tropical Medicine, Volume 10, Issue 8, 2017, Pages 792-801, ISSN 1995-7645, https://doi.org/10.1016/j.apjtm.2017.07.024.
(https://www.sciencedirect.com/science/article/pii/S1995764517304790)

7. Suman Chandra, Shabana Khan, Bharathi Avula, Hemant Lata, Min Hye Yang, Mahmoud A. ElSohly, Ikhlas A. Khan, "Assessment of Total Phenolic and Flavonoid Content, Antioxidant Properties, and Yield of Aeroponically and Conventionally Grown Leafy Vegetables and Fruit Crops: A Comparative Study", *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, Article ID 253875, 9 pages, 2014. https://doi.org/10.1155/2014/253875

8. 27.pdf (globalresearchonline.net)

9. Basak, Piyali & Adhikary, Tathagata & Das, Pratik & Biswas, Shreya. (2018). Phytochemical analysis and comparative study of antibacterial effect of turmeric extracts using different solvent.. IOP Conference Series: Materials Science and Engineering. 410. 012018. 10.1088/1757-899X/410/1/012018.

10. Tolosa, L., Donato, M. T., & Gómez-Lechón, M. J. (2015). General Cytotoxicity Assessment by Means of the MTT Assay. *Methods in molecular biology (Clifton, N.J.), 1250*, 333–348. https://doi.org/10.1007/978-1-4939-2074-7_26

11. PubChem (nih.gov)

12. ADMETlab 2.0 (scbdd.com)

13. BIOVIA Discovery Studio - BIOVIA - Dassault Systèmes® (3ds.com)

14. AutoDock Vina (scripps.edu)

15. RCSB PDB: Homepage

16. Das, P., Majumder, R., Mandal, M. & Basak, P. (2020). In-Silico approach for identification of effective and stable inhibitors for COVID-19 main protease (Mpro) from flavonoid based phytochemical constituents of Calendula officinalis. *Journal of Biomolecular Structure and Dynamics*, 1–16. https://doi.org/10.1080/07391102.2020.1796799 [Taylor & Francis Online], [Google Scholar]

Chapter 5

Results and Discussion

5. Results & Discussion:

5.1. Phytochemical Screening of whole *A. marina* leaf extract

The presence of phytochemicals was confirmed by performing the phytochemical screening using 40% ethanolic extract of A. marina whole leaves. It suggests that there may be some kind of medicinal property in the extract.

Table - 5.1. Phytochemical Screening of A. marina whole leaf extract

Sl. No.	Screening Tests for Phytochemicals	A. Marina leaf extract
1	Phenols	Present
2	Flavonoids	Present
3	Reducing Sugar	Present
4	Alkaloids	Present
5	Tannins	Present
6	Saponins	Present

5.2. Total Phenolic Content (TPC)

The 'Folin–Ciocalteu Reagent' is sensitive to the presence of polyphenols and produces blue coloured complex in presence of polyphenols. The principle of F-C-assay stands on the transfer of electrons in an alkaline medium, from phenols to phosphomolybdic/phosphotungstic acid complexes; this is the reason behind the formation of molybdenum-tungsten blue. Electrons act as the reducing equivalents in the reaction. Here FCR is reduced in reaction with phenolics. [1]

Phenolic compounds have at least one or more hydroxyl groups on their aromatic skeleton. Phenols possess more than one phenolic hydroxyl group attached to one or more benzene ring that construct the skeleton. [2] These are common constituents of different plants, vegetables, spices, fruits, wines, chocolates, tea, coffee etc. [3]

Phenolic compound were previously studied as they contributed to the coloration and taste of food, but recently they have been proven as bioactive possessing important functions like anti-oxidative, anti-microbial, and anti-carcinogenic activities. The phenolic class of compounds consists of numerous compounds, ranging from simple (monocyclic) phenolic acids to flavonoids. Flavonoid itself can be divided into many classes like - flavone, flavanone,

flavonol, anthocyanin, isoflavone, etc. Anthocyanins (a group of polyphenols) are the biggest group of water-soluble natural pigments, forming the vibrant colour of many fruits and flowers. [4]

Anticancer activity of phenolic compounds is contributed to its ability to: (i) trigger cell cycle arrest; (ii) regulate ROS levels; (iii) inhibit oncogenic signalling cascades which in turn control cascades that controls cell proliferation and apoptosis; (iv) heighten the chance to differentiate and transform into normal cells; (v) promote tumour suppressor proteins such as p53. [5]

Our experimental data showed that the TPC in A. marina whole leaf extract was 187.437 µg/mL Gallic acid equivalents (GAE). A standard curve of Gallic Acid (as shown below in Fig. 5.1.) was used for the estimation of total phenolic content in A. Marina whole leaf extract.

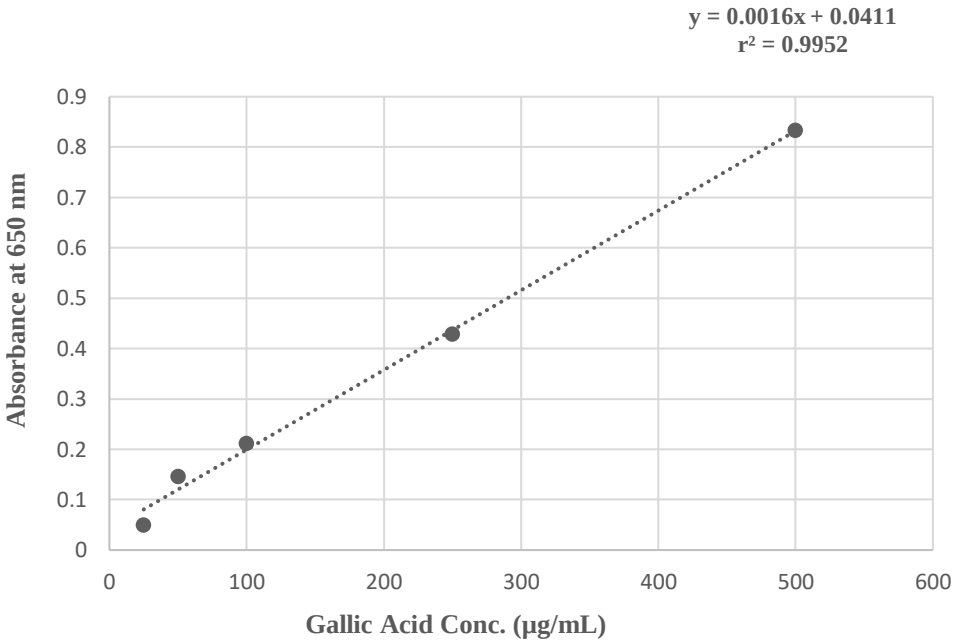

Fig.5.1. Standard Curve for Folin-Ciocalteu Assay

5.3. Total Flavonoid Content (TFC)

Flavonoids are one of the most commonly available group of phenolic compounds, found in plant bodies. They perform many beneficial functions inside human body, such as, anti-oxidant activity, anti-inflammatory activity, anti-bacterial & anti-viral effects, anti-allergenic effect, as well as they possess anti-mutagenic and anti-cancer properties. [6]

Calculating our experiment data it was seen that the total flavonoid content in A. marina whole leaf extract was 177.2 µg/mL. A standard curve of quercetin (Fig 5.2.) was prepared for the estimation of TFC. The result pointed out the fact that A. marina mangrove plant leaf extract

is a rich source for flavonoids. The high TFC of A. marina leaf extract can induce cell cycle blocking, bring forth apoptosis of cancerous cells, showing its importance in anti-cancer researches. [7]

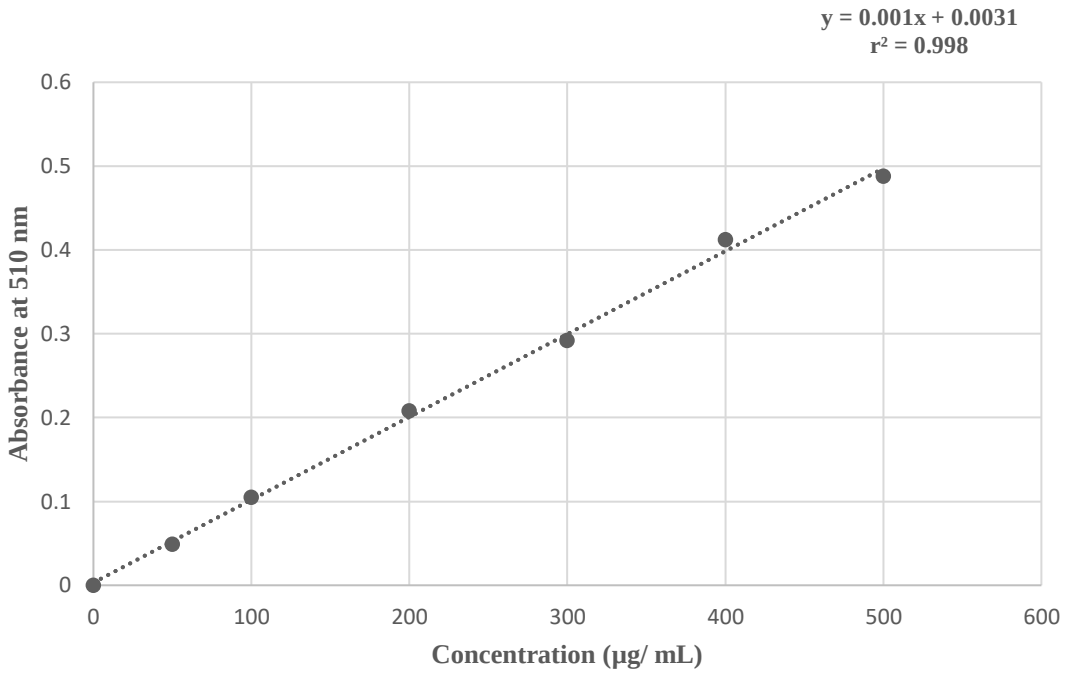

Fig.5.2. Standard Curve of Quercetin

So, the experimental results of total phytochemical contents (total phenolic content & total flavonoid content) of A. marina whole leaf extracts are presented in the table 5.2 as follows –

Table 5.2. Quantitative Analysis of Phytochemicals

Phytochemical	**Concentration (µg/mL)**
Phenols	187.437
Flavonoids	177.2

5.4. Antibacterial Assay

In this experiment after 24 h, very small zones of inhibition are observed in both strains of bacterial culture Petri dish (Fig 5.4.).

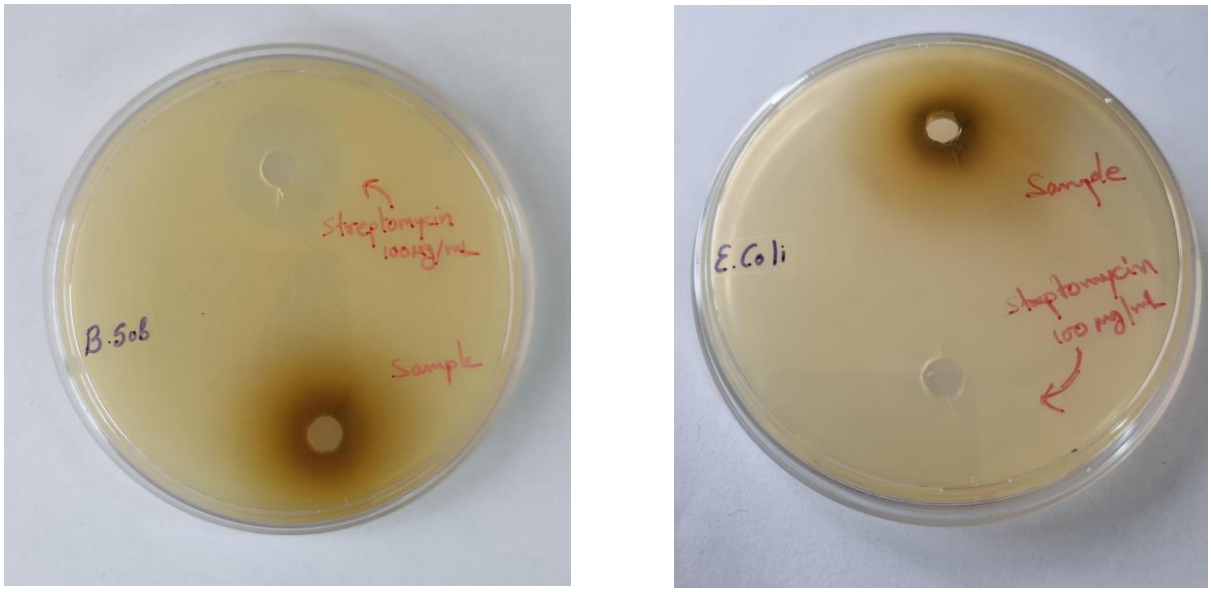

Fig.5.3. Antibacterial activity of A. marina leaf extract showing zone of inhibition against B. subtilis and E. coli respectively

5.5. Antioxidant activity - by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) Assay

Free radical scavenging activity is measured using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) as a free radical. Our data (Fig. 5.4) showed that A. marina whole leaf extract contained 60.234% to 80.734% DPPH free radical scavenging activity at the treatment concentration of 50µg/mL to 100µg/mL. Free radical activity is also compared with ascorbic acid at same concentrations as seen in Fig 5.4.

Our result suggested that A. marina mangrove leaf extract can be a good option for its antioxidant activity in case of inhibiting proliferation of cancer cells without affecting other surrounding normal cells.

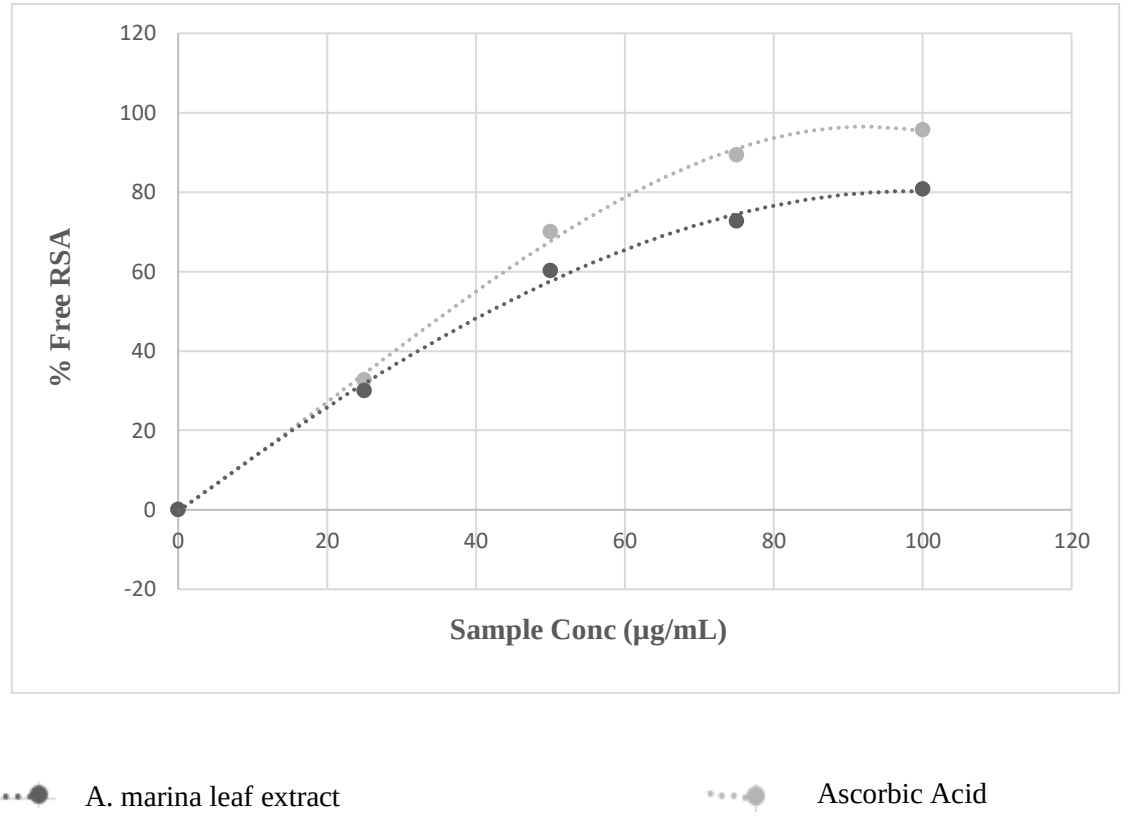

Fig.5.4. Antioxidant Activity of A. marina Whole Leaf Extract and Ascorbic Acid

5.6. Anti-inflammatory activities – by Bovine Serum Albumin Assay (BSA)

The denaturation of protein is one of the main causes behind inflammation. The result showed (Fig. 5.5.) different concentrations of A. marina leaf extract protected the Bovine Serum Albumin (BSA) against heat induced denaturation.

At 25 µg/ml concentration the % anti-denaturation activity is 73.30 i.e. the highest score here. If we follow the curve presented in Fig. 5.5, we can easily see that along with the rise of leaf extract concentration, the percentage of anti-denaturation activity has decreased. So the percentage of BSA protection against heat was increased with decreasing concentration of A. marina leaf extract.

Therefore it can be seen that the A. marina leaf extract indeed possess a very good anti-denaturation property at its lowest concentration.

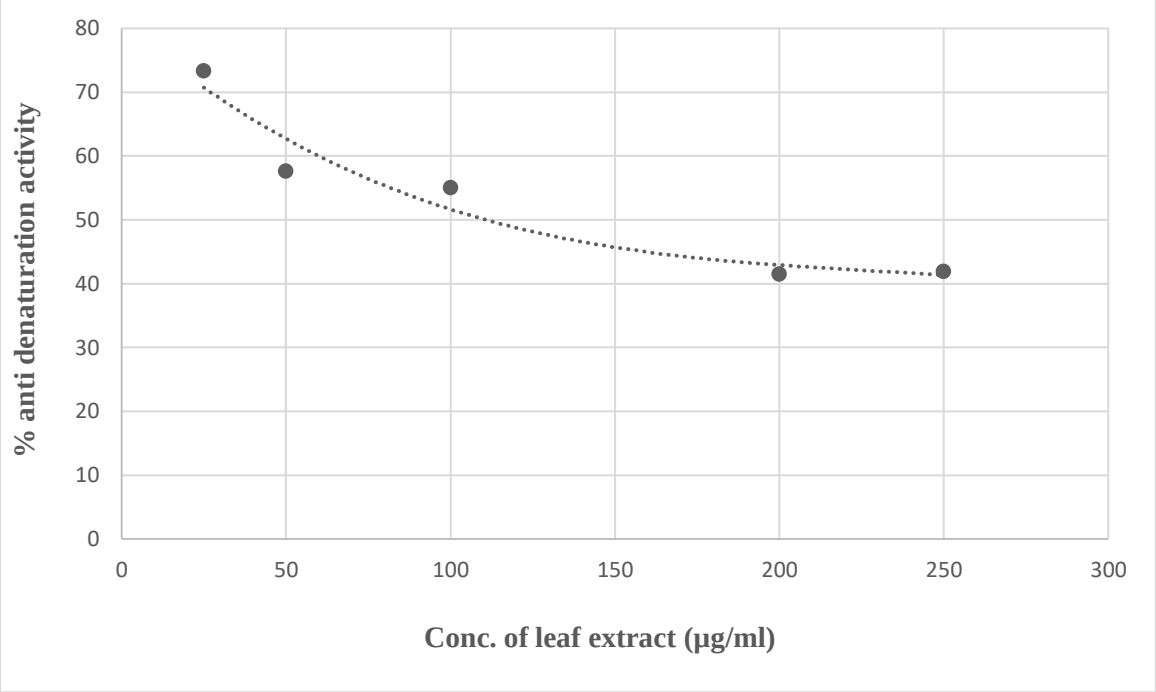

Fig.5.5. Anti-inflammatory Activity of A. marina Whole Leaf Extract

5.7. Cytotoxic test – MTT assay

Dose dependent cytotoxicity of A. marina leaf extract was evaluated against normal non-cancerous mouse fibroblast cell line L929 by conducting MTT assay.

The MTT assay was performed on L929 cells after 24 h of treatment with A. marina whole leaf extract of different concentrations. Our experimental data showed (Fig. 5.6) that different concentration of A. marina leaf extract promoted significant growth of the fibroblast (L929) cells. We can see from the graphical representation of the experimental data (fig. 5.6.) that at 25µg/L concentration of A. marina leaf extract, highest cell proliferation has occurred. At 500µg/mL leaf extract concentration, lowest percentage of cell viability was seen. So we can say with the decreasing concentration of A. marina leaf extract, the percentage viability of L929 cells increases.

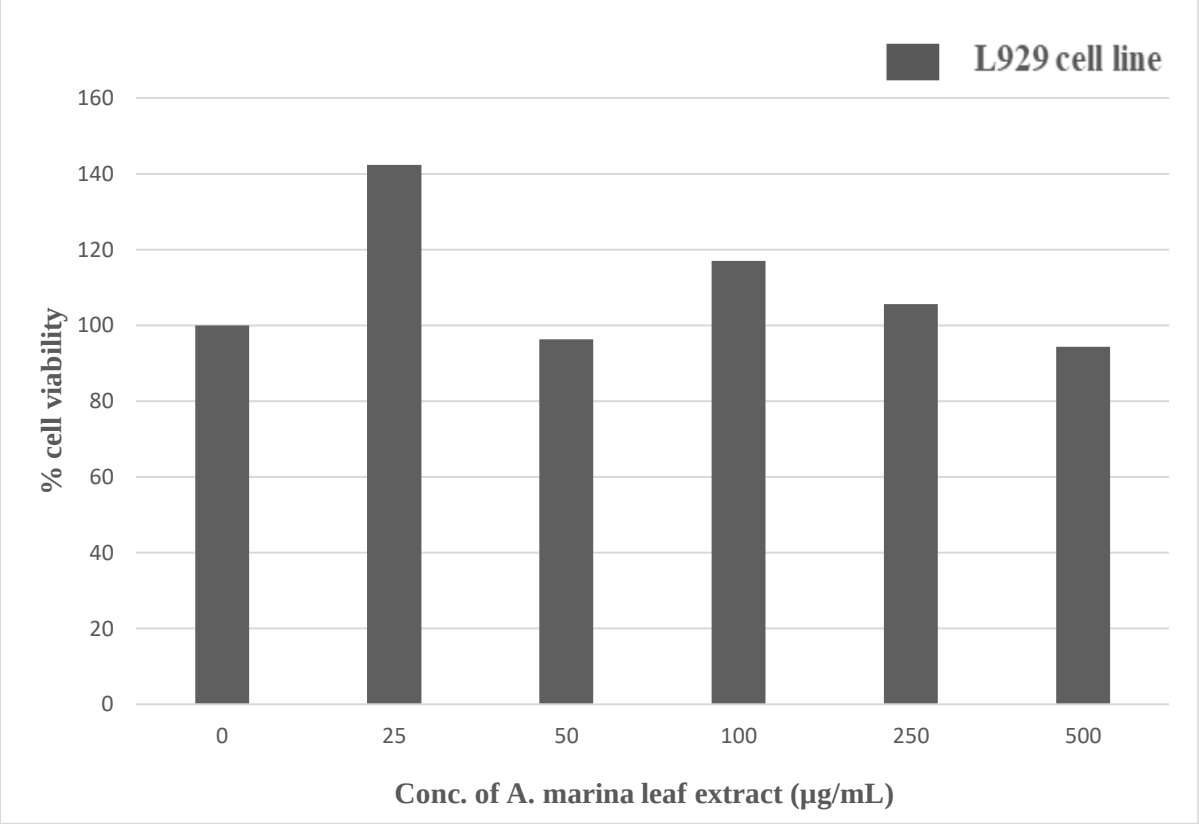

Fig.5.6. Cytotoxic effect of Different Concentrations of A. marina Leaf Extract against L929 Cell Line

5.8. Hemocompatibility Test

According to the accepted standard, % hemolysis score of a test sample which is less than 5, is considered to be highly hemocompatible. Percentage hemolysis value lying within the range of 5 to 10 is considered to be hemocompatible. Percentage hemolysis value greater than 10 implies that the sample is in no way non-hemocompatible.

From our experimental data the calculated value of % hemolysis for different concentrations of A. marina leaf extract varied from 2.52 % to 3.99 %. Thus it can be concluded that the leaf extract samples are highly hemocompatible in nature and so it won't be harmful to prepare a drug from A. marina leaf extract for treating HER2+ breast cancer.

5.9. Virtual Molecular Docking [8]

The previously prepared PDB file of HER2 protein was opened using Autodock Vina software. Afterwards, PDB file of a phytochemical was opened along with the receptor protein.

Then through Torsion Tree path option, root was detected and number of torsions for the ligand was set accordingly. The output result was saved as ligand PDBQT file format.

Now, the protein molecule was selected as macromolecule; here the colour of the protein molecule faded and the output file was saved as protein PDBQT file format.

From Grid option, Grid Box of the following specifications was constructed-

size_x = 126, size_y = 82, size_z = 126; center_x = 55.9, center_y = 87.7, center_z = 78.5

Now the output grid dimension file was saved as a TXT file format.

Using that grid dimension TXT file as reference, a new file named Configuration file (txt format; created using notepad application) was created specifying the following details-

receptor = protein.pdbqt; ligand = ligand.pdbqt; size_x = 126; size_y = 82; size_z = 126; center_x = 55.9; center_y = 87.7; center_z = 78.5; energy_range = 4; exhaustiveness = 8

Now Command Prompt was opened and the specific command was put in it to run the docking process of receptor with the selected ligand.

After some times when the whole program was run, an Output file of PDBQT format containing the docked result was produced.

Then, Discovery Studio was opened and that output PDBQT file and protein PDBQT file were dragged and dropped respectively inside the open window.

After selecting Receptor-Ligand-Interaction option, the protein file was defined as receptor and Hetatm was defined as ligand.

Now the end result for docking i.e. the detailed 2D diagram of receptor-ligand complex structure could be seen in the Discovery Studio window along with its 3D format.

Table - 5.3. Docking Scores of standard drugs for HER2 Positive Breast Cancer with HER2 Receptor

Sl. No.	Name of the Drug	Affinity with HER2 (kcal/mol)
1.	Lapatinib	-7.6
2.	Neratinib	-8.1

Lapatinib and Neratinib are considered as the standard anti-cancer drugs used for the treatment of HER2+ breast cancer.

As the docking score of Neratinib and lapatinib with HER2 was found respectively -8.1 and -7.6 kcal/mol, we considered all the docking scores between the range of -7.6 to -8.1 as good and acceptable docking scores for the selected ligands.

Score beyond -8.1 (i.e. less than -8.1) was clearly regarded as even better score than the standard drugs

The scores greater than -7.6 was regarded as poor docking score indicating less affinity with the receptor.

The following section shows the interaction of the two standard drugs for HER2 positive breast cancer with HER2 receptor protein respectively.

Fig.5.7 shows the standard drug, Lapatinib is in interaction with HER2 Protein Receptor and Fig.5.8 portrays the standard Drug, Neratinib is in interaction with HER2 Protein Receptor.

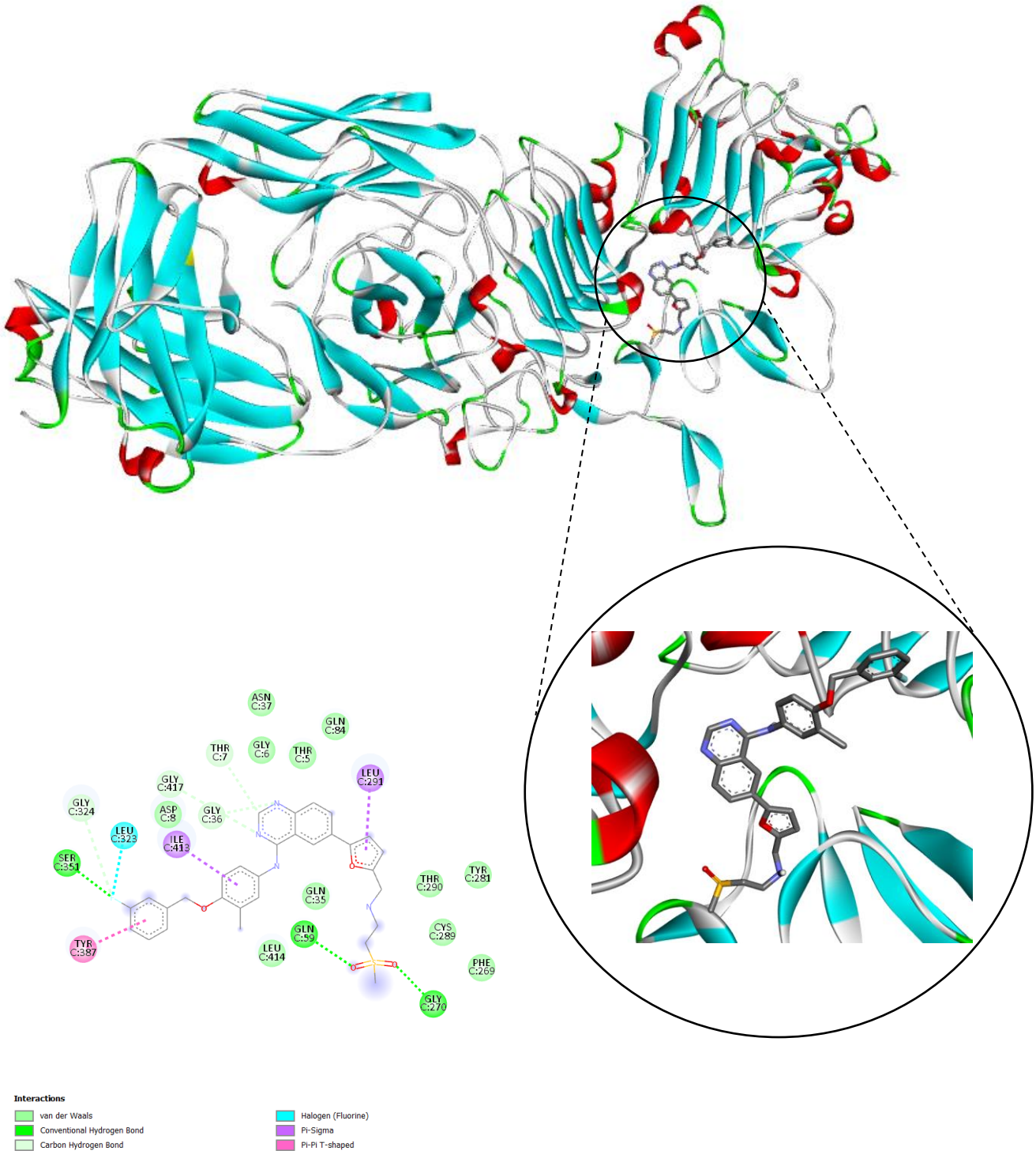

Fig.5.7. Standard Drug, Lapatinib in interaction with HER2 Protein Receptor

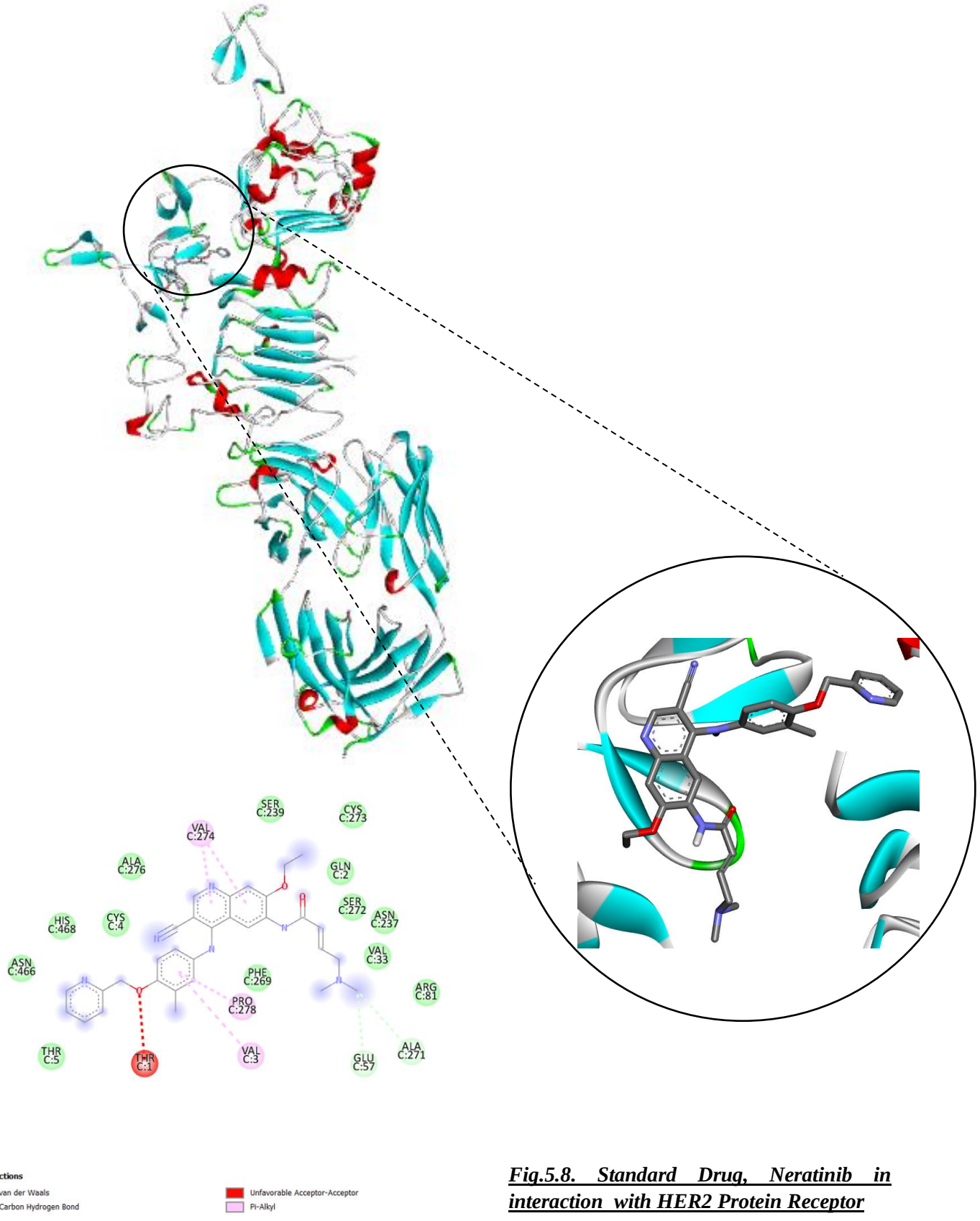

Fig.5.8. Standard Drug, Neratinib in interaction with HER2 Protein Receptor

Table - 5.4. Docking Scores of A. marina Leaf Phytochemicals with HER2 Receptor

Sl No.	*A. Marina* leaf Phytochemicals	Affinity with HER2 (kcal/mol)	Comment
1	Squalene	-4.5	NA
2	2(R),3(S)-1,2,3,4-Butane tetrol	-4.7	NA
3	n-Hexadecanoic acid	-4.2	NA
4	Hexadecanoic acid, ethyl ester	-4.0	NA
5	3,7,11,15 – tetramethyl – 2 – hexadecane – 1 – ol	-4.5	NA
6	Phytol	-4.8	NA
7	(E)-9-Octadecenoic acid ethyl ester	-4.3	NA
8	Dodecanoic acid/lauric acid	-4.0	NA
9	Octadecanoic acid,2-methyl-,methyl ester	-4.5	NA
10	cis-9-Hexdecenal	-4.1	NA
11	D-Allose	-5.9	NA
12	Nonanoic acid	-3.9	NA
13	Quercetin	-8.2	ACCEPTED
14	Kaempferol	-7.4	NA
15	Lupeol	-8.7	ACCEPTED
16	Betulin	-7.9	ACCEPTED
17	α-amyrin	-9.3	ACCEPTED (Highest Score)
18	Linolenic acid	-4.6	NA
19	Stearic acid	-4.3	NA
20	Myristic acid	-3.6	NA
21	Geniposidic Acid	-6.9	NA
22	Mussaenoside	-6.9	NA
23	Syringaresinol	-7.5	NA
24	5-hydroxy-4, 7-dimethoxyflavone	-7.1	NA
25	4',5-Dihydroxy-3',5'-dimethoxyflavone	-7.5	NA
26	3’,4’,5-trihydroxy-7-methoxyflavone	-7.9	ACCEPTED
27	ß-sitosterol	-7.7	ACCEPTED
28	Ergost-6,22-diene-5,8-epidioxy-3ß-ol	-7.5	NA
29	Palmitic acid	-4.4	NA
30	p-methoxy cinnamic acid	-5.8	NA

NA = Not Accepted (i.e. having Poor affinity with the receptor)

From Table 5.4 it could be easily seen that 6 out of the 30 phytochemicals (ligands) might be accepted as potential substitution of the standard drugs for HER2+ breast cancer.

Among those 6 ligands, α-amyrin (score -9.3), Lupeol (score -8.7) and Quercetin (score -8.2), these 3 ligands had shown even better affinity with HER2 than the standard drugs.

Among these 3 ligands, α-amyrin has the highest affinity with HER2 protein receptor. So this can be considered as the best among the lot.

Therefore α-amyrin can be considered as a natural substitution of Lapatinib and Neratinib, the standard drugs for HER2+ breast cancer treatment.

In the next section the results of the docking i.e. the interactions of all the 6 ligands with HER2 receptor have been portrayed.

In the Fig. 5.9 Ligand, α-amyrin (docking score -9.3) is seen in interaction with HER2 protein.

Fig. 5.10 shows the ligand, Lupeol (docking score -8.7) in interaction with HER2.

Fig.5.11 portrays the ligand, Quercetin docking score -8.2) in interaction with HER2 breast cancer protein.

Fig.5.12 shows the ligand, 3', 4', 5-trihydroxy-7-methoxyflavone (docking score -7.9) in interaction with HER2.

In the Fig.5.13 ligand ß-sitosterol (docking score -7.7) is seen in interaction with HER2 protein.

Lastly Fig.5.14 shows the ligand, Betulin (docking score -7.9) is in interaction with HER2 receptor protein.

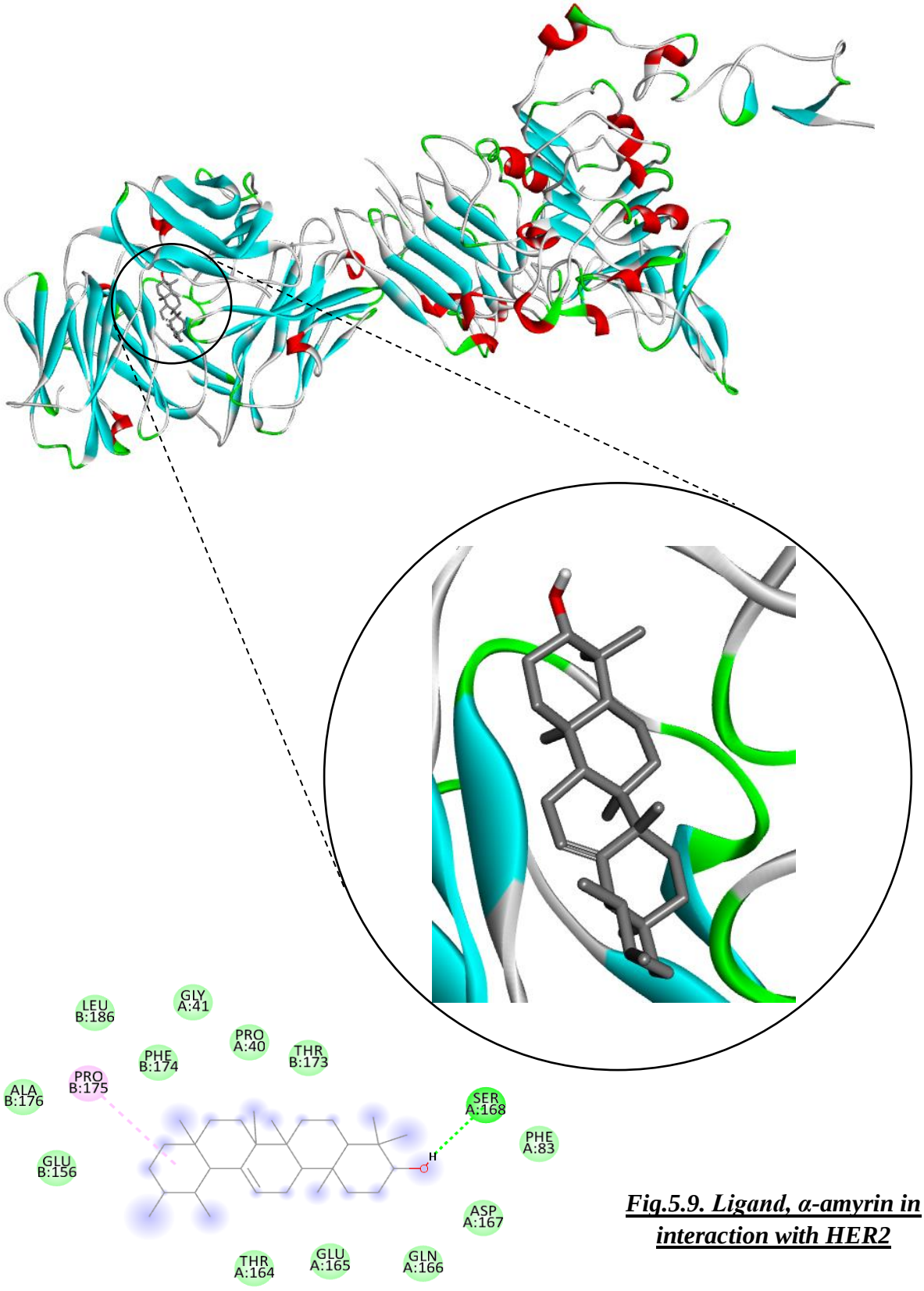

Fig.5.9. Ligand, α-amyrin in interaction with HER2

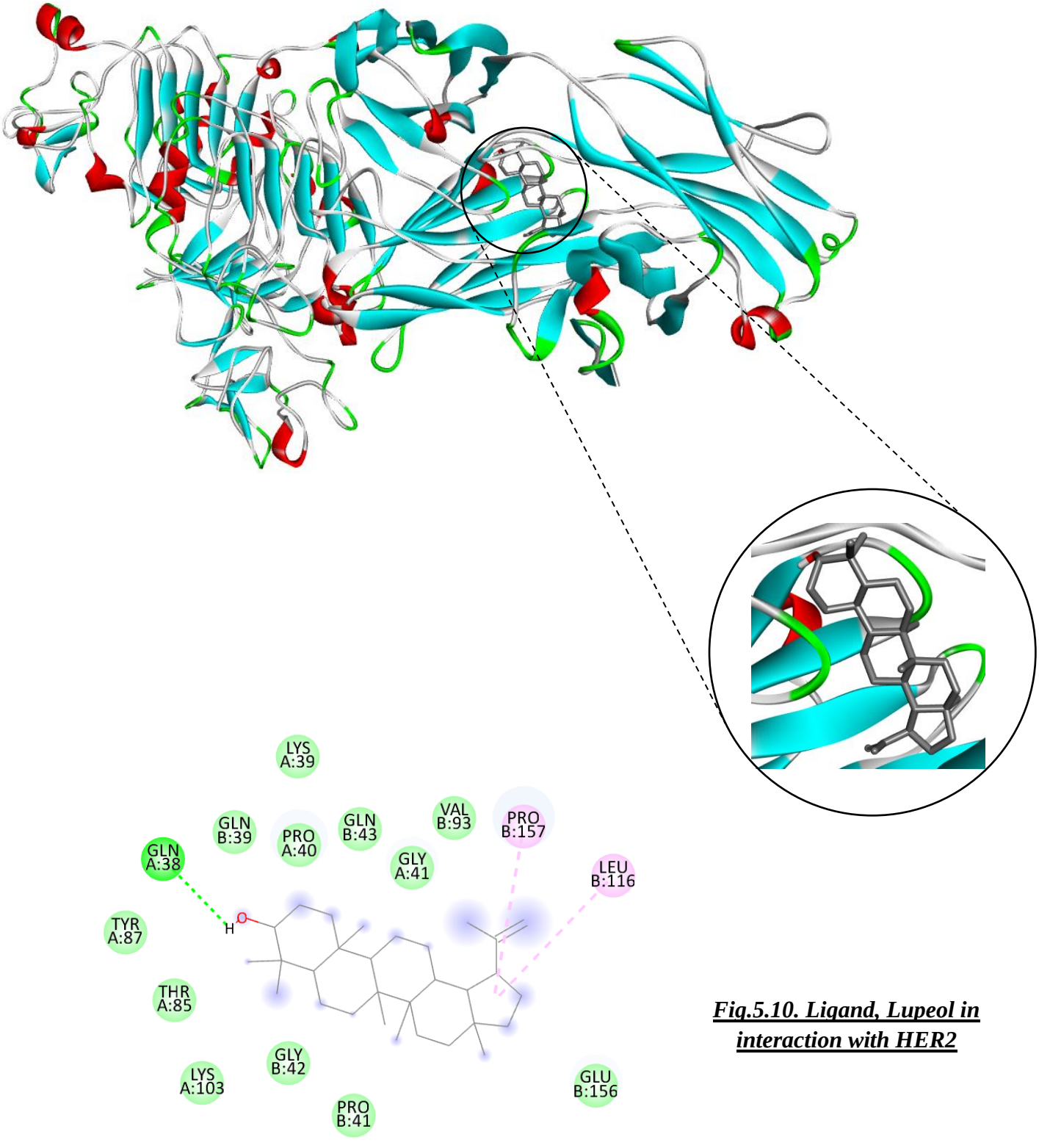

Fig.5.10. Ligand, Lupeol in interaction with HER2

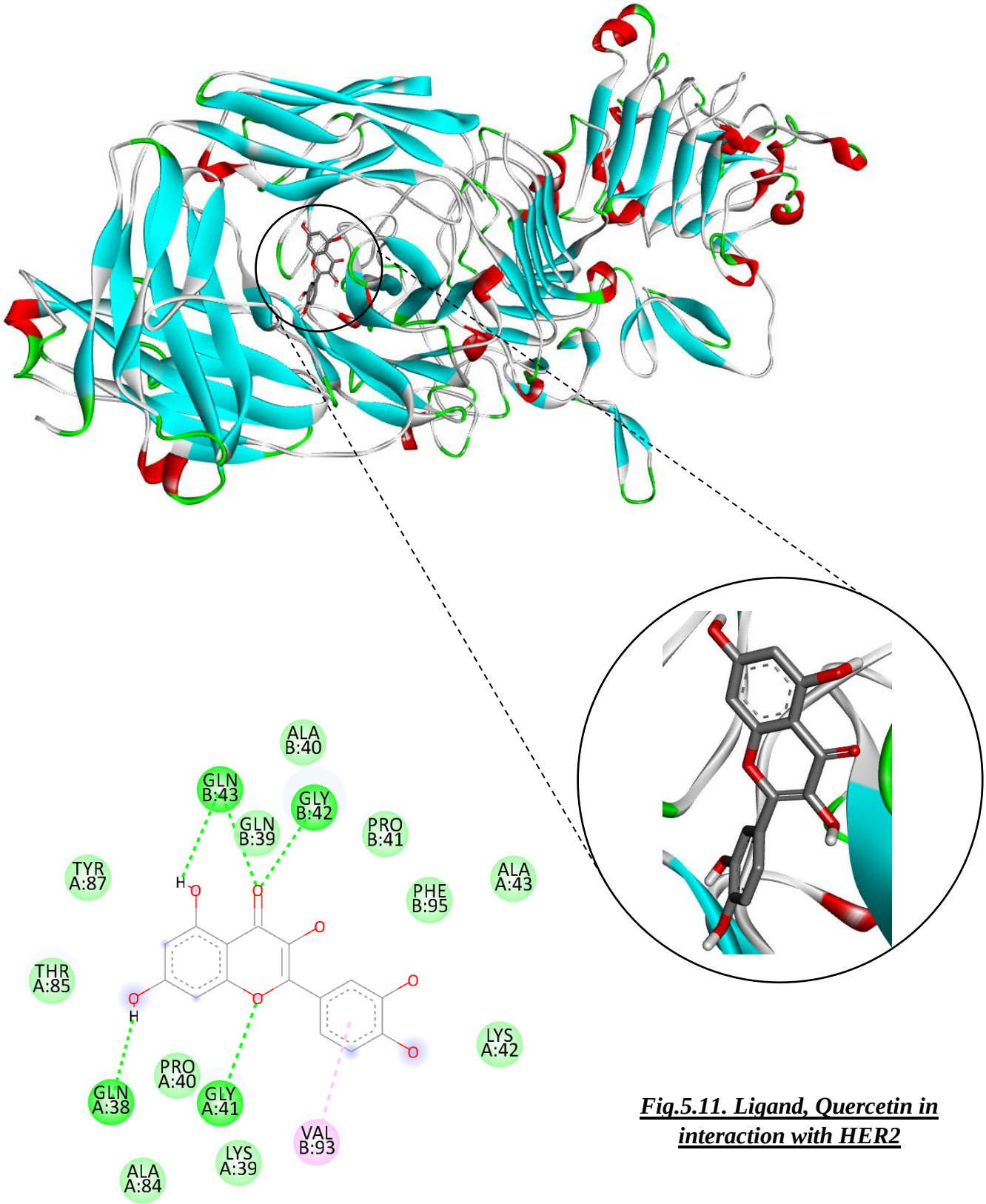

Fig.5.11. Ligand, Quercetin in interaction with HER2

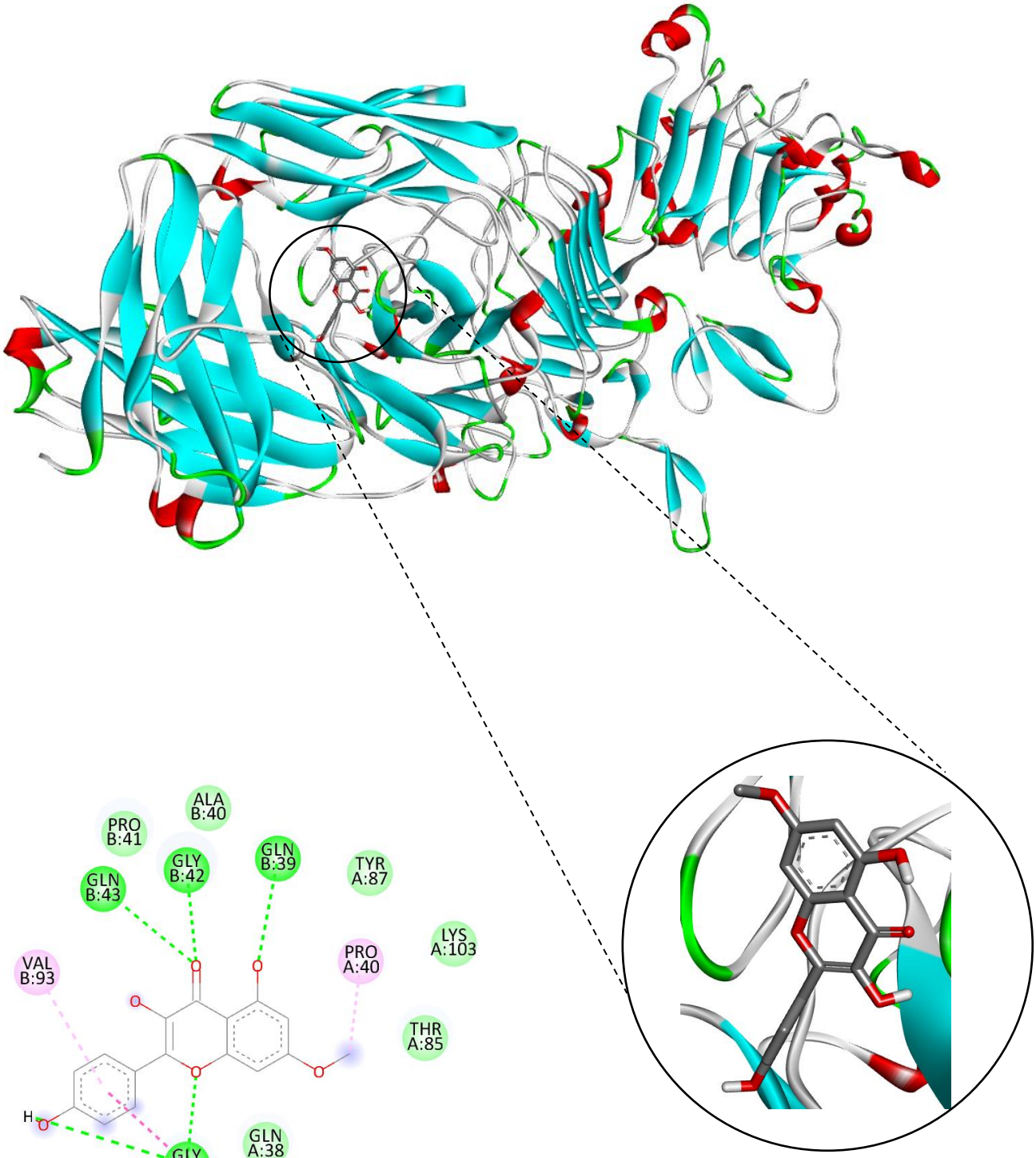

Fig.5.12. Ligand, 3', 4', 5-trihydroxy-7-methoxyflavone in interaction with HER2

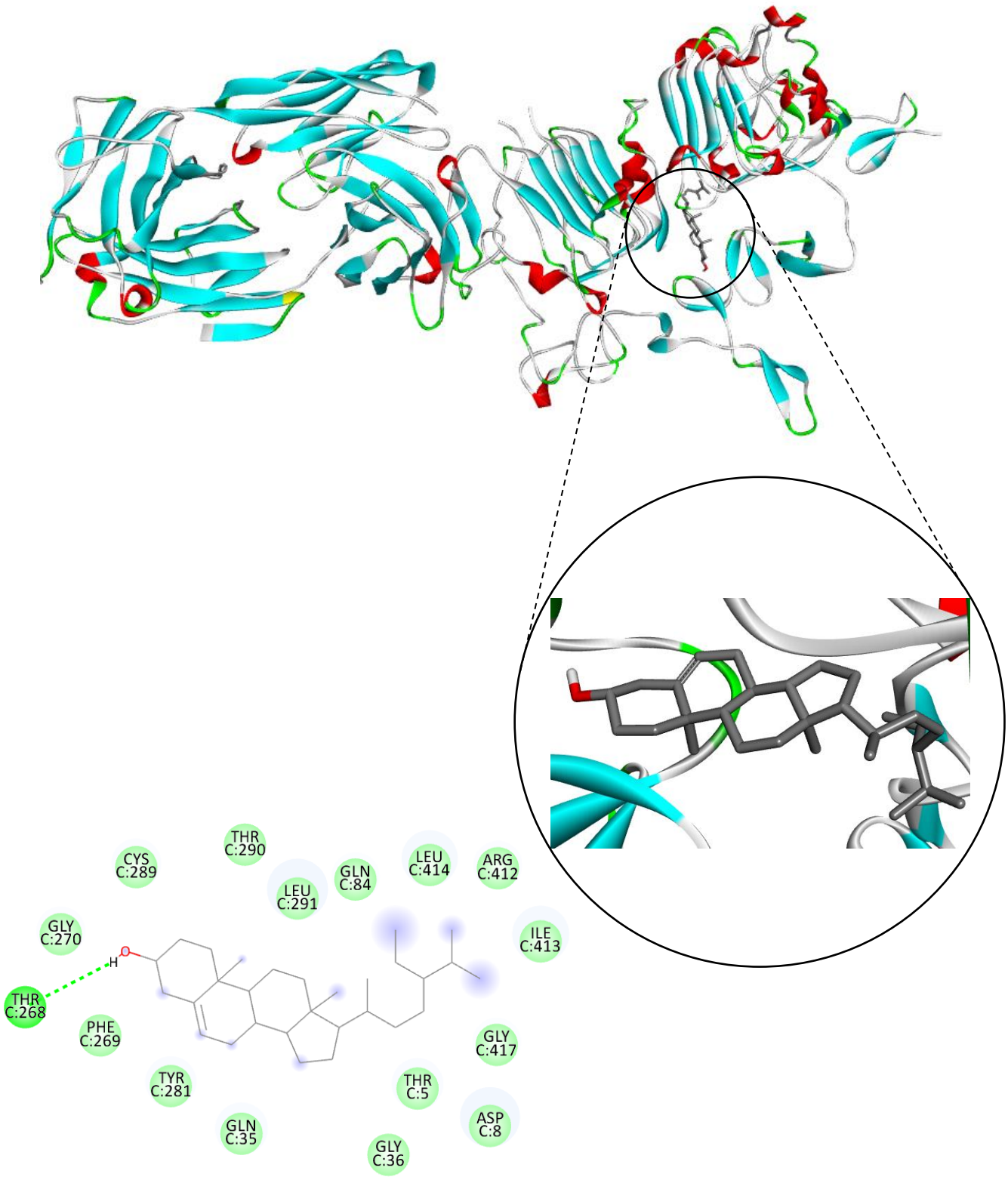

Fig.5.13. Ligand, ß-sitosterol in interaction with HER2

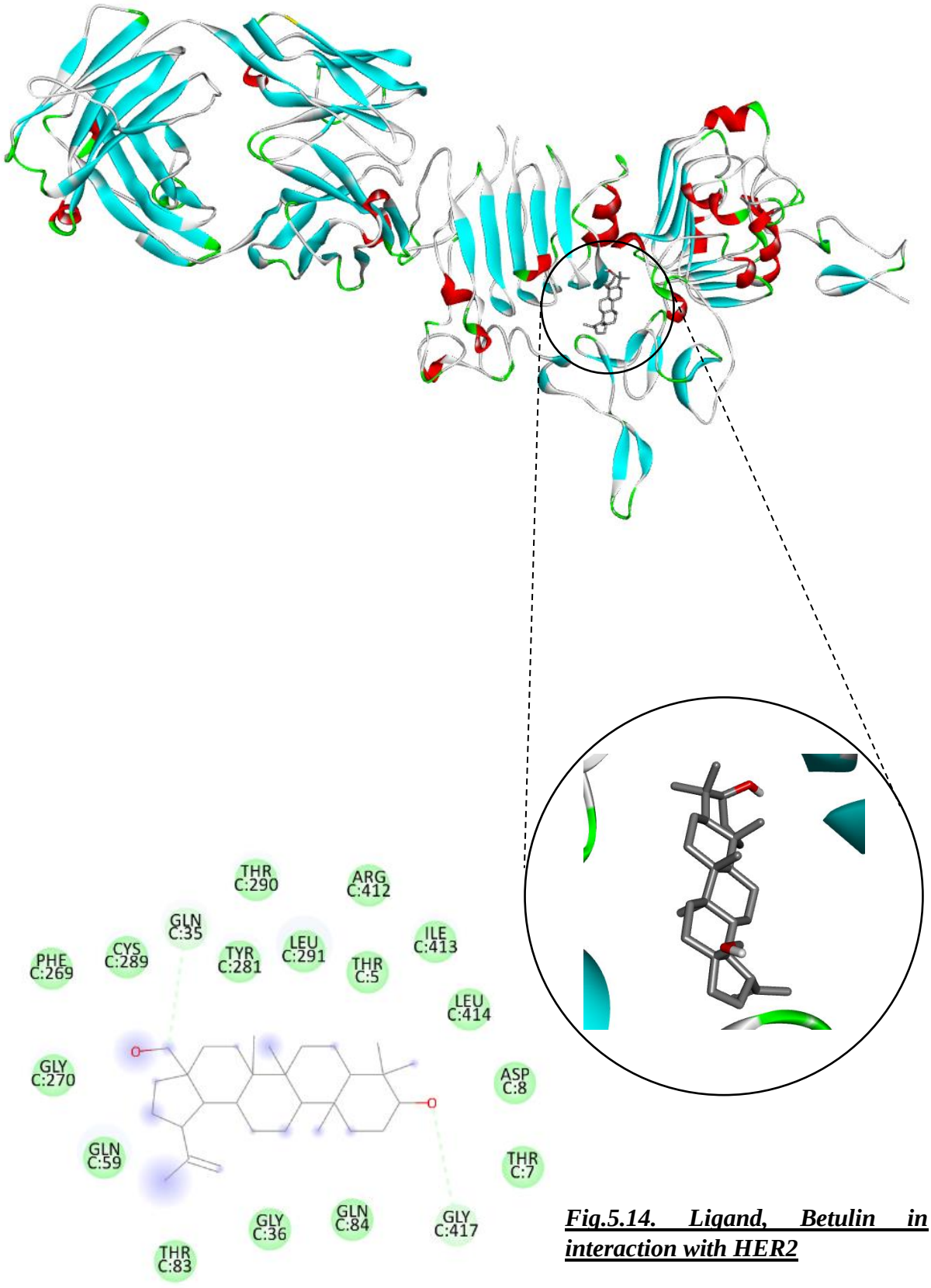

Fig.5.14. Ligand, Betulin in interaction with HER2

References:

1. Swain, T. and Hillis, W.E. (1959) The Phenolic Constituents of Prunus domestica. I.—The Quantitative Analysis of Phenolic Constituents. Journal of the Science of Food and Agriculture, 10, 63-68. http://dx.doi.org/10.1002/jsfa.2740100110

2. Phenolic Compound - an overview | ScienceDirect Topics

3. Perron, N.R. and Brumaghim, J.L. (2009) A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. Cell Biochemistry and Biophysics, 53, 75-100. Hdoi:10.1007/s12013-009-9043-x

4. E. Ibañez, F.J. Señoráns, in Encyclopedia of Analytical Science (Second Edition), 2005

5. Preethi G. Anantharaju1, Prathima C. Gowda2, Manjunatha G. Vimalambike3 and SubbaRao V. Madhunapantula;"An overview on the role of dietary phenolics for the treatment of cancers"; Nutrition Journal (2016) 15:99 DOI 10.1186/s12937-016-0217-2

6. Malta, L.G. & Liu, R.H.. (2014). Analyses of Total Phenolics, Total Flavonoids, and Total Antioxidant Activities in Foods and Dietary Supplements. 10.1016/B978-0-444-52512-3.00058-9.

7. Kuntz S, Wenzel U, Daniel H. Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. Eur J Nutr 1999;38:133–42.

8. Das, P., Majumder, R., Mandal, M. & Basak, P. (2020). In-Silico approach for identification of effective and stable inhibitors for COVID-19 main protease (Mpro) from flavonoid based phytochemical constituents of Calendula officinalis. *Journal of Biomolecular Structure and Dynamics*, 1–
16. https://doi.org/10.1080/07391102.2020.1796799 [Taylor & Francis Online], [Google Scholar]

Chapter 6

Conclusion & Future Plan of Work

6.1. Conclusion:

Our study showed that *A. marina* whole leaf extract has a significant amount of antioxidant and anti-inflammatory properties. *A. marina* leaf extract also promoted the growth of non-tumour non-cancerous fibroblastic cells.

All these pharmacological activities of *A. marina* leaf extract may be because of the presence of secondary metabolites like phenolic compounds, flavonoids content in its leaf. Antioxidant property of *A. marina* extract might inhibit breast cancer cells by inducing selective apoptosis in the breast cancer cells.

Crude *A. marina* leaf extract showed zone of inhibition against both gram positive and gram negative bacteria proving it possesses antibacterial property.

In silico docking study suggested that *A. marina* leaf specific phytochemical compounds can inhibit breast cancer protein (here we have selected HER2 as the receptor protein) thus can prevent cancerous cell proliferation.

So, considering both in silico and in vitro study of *A. marina* leaf phytochemicals, we can conclude that this mangrove leaf extract can inhibit the growth and proliferation of cancerous cells at the same time promoting the growth and proliferation of normal cells. Therefore we can say that if anticancer drugs are to be designed from *A. marina* leaf phytochemicals, they can be very effective in treating breast cancer.

Still much more future studies and researches in this regard are necessary to actualize the idea of developing a therapeutic agent from A. marina leaf phytochemicals, which will effectively fight against breast cancer.

6.2. Future Plan of Work:

- In silico docking of *A. marina* leaf phytochemicals against Estrogen and progesterone receptor proteins.
- In vitro anticancer study of the different concentration of *A. marina* leaf sample to be cultivated against different breast cancer cell lines (like BT474, MCF-7 & T47D etc.).
- Isolation and purification of the antitumor bioactive compounds from *A. marina* whole leaf extract.
- Green Synthesis of biogenic silver nanoparticles (AgNPs) from *A. marina* leaf extract.
- Evaluation of cytotoxic effect of AgNPs against breast cancer cell lines.
- Calculation of IC50 and LD50.
- Investigation of the detailed mechanism of action of bioactive agents against breast cancer.
- Evaluation of the effect of bioactive agents on cell cycle and apoptosis.
- Performing in-vivo experiment with nude mice model.
- Investigating the effect of *A. marina* extract on the immune system.
- Development of a non-invasive, patient-friendly drug delivery system for administrating antitumor bioactive agents targeting breast tumours.
- Further anticancer studies on other plant parts like flower, bark, etc. of A. marina mangrove.