IN-SILICO AND *IN-VITRO* EVALUATION OF *ANNONA MURICATA* LEAF EXTRACT CONJUGATED WITH DOXORUBICIN VIA LIPOSOMAL DRUG DELIVERY SYSTEM AGAINST (MDA-MB-231) TRIPLE NEGATIVE BREAST CANCER CELL LINE

A Dissertation submitted to

THE TAMIL NADU DR.M.G.R MEDICAL UNIVERSITY
CHENNAI - 600 032

In partial fulfillment of the requirements for the award of the Degree of

MASTER OF PHARMACY

IN

BRANCH-VI-> PHARMACOLOGY

Submitted by

PRAVEEN KUMAR K N

REGISTRATION No. 261926151

Under the guidance of

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CERTIFICATES

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PRINCIPAL

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Othakkalmandapam, Coimbatore-641032, Tamilnadu, India

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RESEARCH GUIDE

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I hereby declare that this dissertation work entitled **"*IN-SILICO* AND *IN-VITRO* EVALUATION OF *ANNONA MURICATA* LEAF EXTRACT CONJUGATED WITH DOXORUBICIN VIA LIPOSOMAL DRUG DELIVERY SYSTEM AGAINST (MDA-MB-231) TRIPLE NEGATIVE BREAST CANCER CELL LINE"** submitted by me, in partial fulfillment for the award of the degree of **Master of Pharmacy in Pharmacology** to **The Tamilnadu Dr. M.G.R. Medical University, Chennai** is the result of my original and independent research work carried out under the guidance of **Dr. C. SENTHIL KUMAR, M.Pharm., PGDCR., Ph.D. Department of Pharmacology, Karpagam College of Pharmacy, Coimbatore** during the academic year 2020-2021

The work is original and the dissertation either in part or full has not been submitted by me or any other person to any University/Institute in any part of thesis/ dissertation/ monograph.

I hereby further declare that the **Department of Pharmacology, Karpagam College of Pharmacy, Coimbatore** shall have the rights to preserve, use and disseminate this dissertation in print or electronic format for academic or research purpose.

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EVALUATION CERTIFICATE

This is to certify that the dissertation entitled "***IN-SILICO* AND *IN-VITRO* EVALUATION OF *ANNONA MURICATA* LEAF EXTRACT CONJUGATED WITH DOXORUBICIN VIA LIPOSOMAL DRUG DELIVERY SYSTEM AGAINST (MDA-MB-231) TRIPLE NEGATIVE BREAST CANCER CELL LINE"** is a bonafied research work done by **Mr.PRAVEEN KUMAR K N (Reg. No: 261926151)** and submitted to **The Tamilnadu Dr. M.G.R. Medical University, Chennai** in partial fulfillment for the award of the degree of **Master of Pharmacy in Pharmacology** is a bonafied research work carried out during the academic year 2020-2021 by the candidate at the **Department of Pharmacology, Karpagam College of Pharmacy, Coimbatore** and was evaluated by us.

Examination Centre:-

Date:-

Internal Examiner **External Examiner**

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By,

Mr.PRAVEEN KUMAR K N

Reg.No: (261926151)

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LIST OF ABBREVIATIONS

1.	%	Percentage
2.	+	Positive
3.	-	Negative
4.	<	Less Than
5.	>	Greater Than
6.	DNA	Deoxyribo Nucleic acid
7.	RNA	Ribo Nucleic Acid
8.	WHO	World Health Organization
9.	DALYs	Disability Adjusted Life Years
10.	HDI	Human Development Index
11.	ASIR	Age Standardized Incidence Rates
12.	MIR	Mortality-To-Incidence Ratio
13.	DCIS	Ductal Carcinoma In Situ
14.	LCIS	Lobular Carcinoma In Situ
15.	IDC	Invasive Ductal Carcinoma
16.	IBC	Inflammatory Breast Cancer
17.	ILC	Invasion Lobular Carcinoma
18.	CTCs	Circulating Tumor Cells
19.	DTCs	Disseminated Tumor Cells
20.	MICs	Metastasis initiating cells
21.	HER2	Human Epidermal Growth Factor Receptor 2
22.	EMT	Epithelial-to-Mesenchymal Transition
23.	TNBC	Triple-Negative Breast Cancer
24.	ACS	American Cancer society
25.	MRI	Magnetic Resonance Imaging
26.	ER	Estrogen Receptor
27.	PR	Progesterone Receptor
28.	MDR 1	Multi Drug Resistance Protein 1
29.	ATP	Adenosine Tri Phosphate
30.	ABCB1	ATP Binding Cassette sub-family B member-1
31.	Cav-1	Cavin protein or Caveolin-1
32.	AP-2	Adaptor Protein-2
33.	P-gp	P-Glycoprotein
34.	CYP450	Cytochrome P450
35.	PM	Plasma Membrane
36.	CCV	Clathrin-Coated Vesicle
37.	GTPase	Guanine Tri Phosphate
38.	AM	Annona Muricata
39.	AGEs	Acetogenins
40.	EGFR	Epidermal Growth Factor Receptor
41.	CADD	Computer Aided Drug Design
42.	DOX	Doxorubicin
43.	AM	Annona muricata

44.	BC	Breast Cancer
45.	ADMET	Absorption ,Distribution ,Metabolism ,Elimination ,Toxicity
46.	SUV	Small Unilamellar Vesicles
47.	LUV	Large Unilamellar Vesicles
48.	MLV	Multi Lamellar Vesicles
49.	PDB	Protein Data Bank
50.	Ro5	Rule of Five
51.	TPSA	Topological Polar Surface Area
52.	CaCo2	Colorectal Adenocarcinima cell
53.	VD	Volume of Distribution
54.	BBB	Blood Brain Barrier
55.	CNS	Central Nervous System
56.	OCT2	Organic Cation Transporter 2
57.	AMES	Salmonella Typhimurium Reverse Mutation Assay
58.	hERG	Human Ether-a-go-go Gene
59.	LD-50	Lethal Dose-50
60.	LOAEL	Lowest Observed Adverse Effect Level
61.	NOAEL	No Observed Adverse Effect Level
62.	IC-50	Inhibitory Concentration
63.	hERG	Human Ether-a-go-go Gene

1. INTRODUCTION

1. CANCER

Cancer is a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body. Cancer is a generic term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumors and neoplasms.[1]

Cancer can start almost anywhere in the human body, which is made up of trillions of cells. Normally, human cells grow and multiply (through a process called cell division) to form new cells as the body needs them. When cells grow old or become damaged, they die, and new cells take their place.[2]

Sometimes this orderly process breaks down, and abnormal or damaged cells grow and multiply when they shouldn't. These cells may form tumors, which are lumps of tissue. Tumors can be cancerous or not cancerous (benign).

Cancerous tumors spread into, or invade, nearby tissues and can travel to distant places in the body to form new tumors (a process called metastasis). Cancerous tumors may also be called malignant tumors. Many cancers form solid tumors, but cancers of the blood, such as leukemias, generally do not.

Benign tumors do not spread into, or invade, nearby tissues. When removed, benign tumors usually don't grow back, whereas cancerous tumors sometimes do. Benign tumors can sometimes be quite large, however. Some can cause serious symptoms or be life threatening, such as benign tumors in the brain.

1.1 Epidemiology of cancer

GLOBOCAN 2020 estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer. Worldwide, an estimated 19.3 million new cancer cases (18.1 million excluding nonmelanoma skin cancer) and almost 10.0 million cancer deaths (9.9 million excluding nonmelanoma skin cancer) occurred in 2020. The disease is an important cause of morbidity and mortality worldwide, in every world region, and irrespective of the level of human development.[3]

The distribution of all-cancer incidence and mortality according to world region for both sexes combined and separately for men and women. For both sexes combined, one-half of all cases and 58.3% of cancer deaths are estimated to occur in Asia in 2020, where 59.5% of the global population resides. Europe accounts for 22.8% of the total cancer cases and 19.6% of the cancer deaths, although it represents 9.7% of the global population, followed by the Americas' 20.9% of incidence and 14.2% of mortality worldwide. In contrast to other regions, the share of cancer deaths in Asia (58.3%) and Africa (7.2%) are higher than the share of incidence (49.3% and 5.7%, respectively) because of the different distribution of cancer types and higher case fatality rates in these regions.

The global burden of cancer worldwide using the GLOBOCAN 2018 estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer, with a focus on geographic variability across 20 world regions. There will be an estimated 18.1 million new cancer cases (17.0 million excluding nonmelanoma skin cancer) and 9.6 million cancer deaths (9.5 million excluding nonmelanoma skin cancer) in 2018.[4]

They estimate that there will be 18.1 million new cases (17.0 million excluding NMSC) and 9.6 million cancer deaths (9.5 million excluding NMSC) worldwide in 2018. The distribution of all-cancer incidence and mortality according to world area for both sexes combined and separately for men and women. For both sexes combined, it is estimated that nearly one-half of the cases and over one-half of the cancer deaths in the world will occur in Asia in the year 2018, in part because close to 60% of the global population resides there. Europe accounts for 23.4% of the total cancer cases and 20.3% of the cancer deaths, although it represents only 9% of the global population, followed by the Americas' 21% of incidence and 14.4% of mortality worldwide. In contrast to other regions, the shares of cancer deaths in Asia (57.3%) and Africa (7.3%) are higher than the shares of incidence (48.4% and 5.8%, respectively) because of the different distribution of cancer types and higher case fatality rates in these regions.[5]

In 2012 estimated that 14.1 million new cancer cases and 8.2 million cancer deaths occurred in 2012 worldwide. The estimated number of cases and deaths for all cancers combined (excluding nonmelanoma skin cancers) and for 27 specific cancers in men, women and both sexes, together with the corresponding and the cumulative risk.[6]

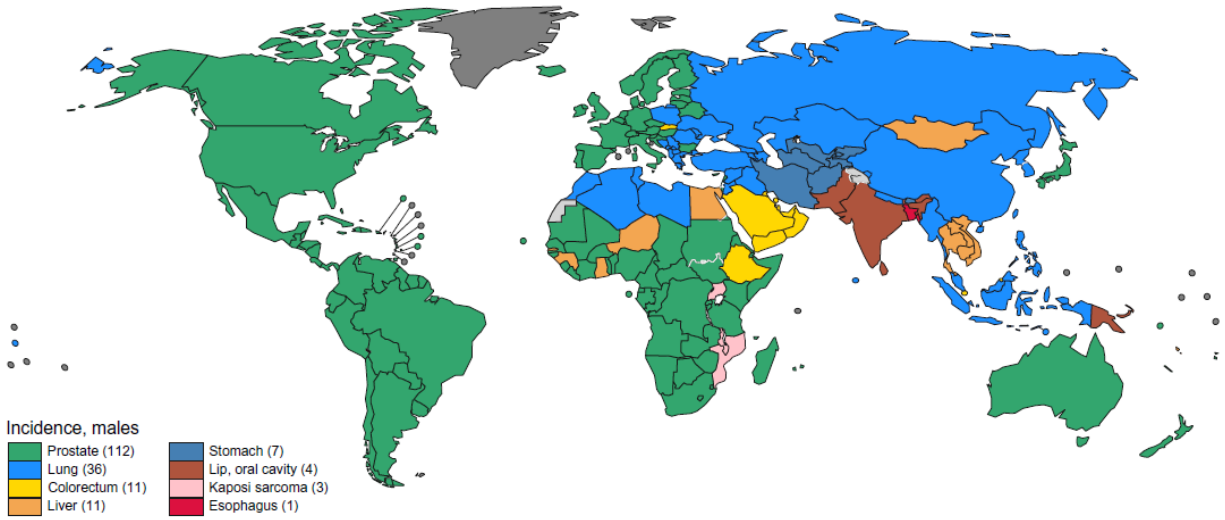


FIGURE 1: EPIDEMIOLOGICAL SURVEY ON MALES IN 2020[7]

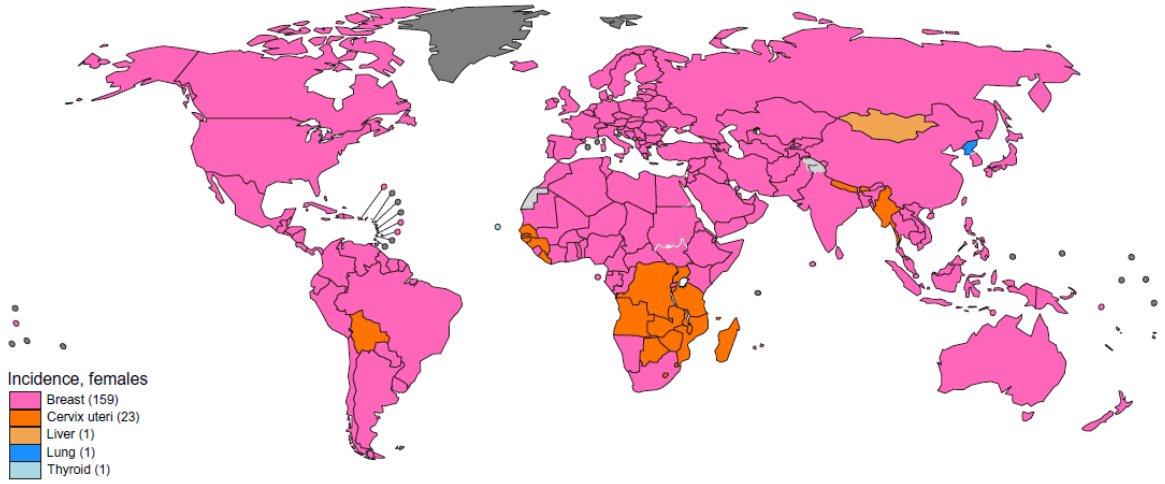


FIGURE 2: EPIDEMIOLOGICAL SURVEY ON FEMALES IN 2020[7]

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020[8]

The most common in 2020 (in terms of new cases of cancer) were:

- Breast (2.26 million cases);
- Lung (2.21 million cases);
- Colon and rectum (1.93 million cases);
- Prostate (1.41 million cases);
- Skin (non-melanoma) (1.20 million cases); and
- Stomach (1.09 million cases).

The most common causes of cancer death in 2020 were:

- Lung (1.80 million deaths);
- Colon and rectum (935 000 deaths);
- Liver (830 000 deaths);
- Stomach (769 000 deaths); and
- Breast (685 000 deaths).

1.2 BREAST CANCER

Cancer occurs when changes called mutations take place in genes that regulate cell growth. The mutations let the cells divide and multiply in an uncontrolled way.

Breast cancer is cancer that develops in breast cells. Typically, the cancer forms in either the lobules or the ducts of the breast. Lobules are the glands that produce milk, and ducts are the pathways that bring the milk from the glands to the nipple. Cancer can also occur in the fatty tissue or the fibrous connective tissue within your breast.

The uncontrolled cancer cells often invade other healthy breast tissue and can travel to the lymph nodes under the arms. The lymph nodes are a primary pathway that help the cancer cells move to other parts of the body.

After skin cancer, breast cancer is the most common cancer diagnosed in women. Breast cancer can occur in both men and women, but it's far more common in women. Substantial support for breast cancer awareness and research funding has helped created advances in the diagnosis and treatment of breast cancer. Breast cancer survival rates have increased, and the number of deaths associated with this disease is steadily declining, largely due to factors such as earlier detection, a new personalized approach to treatment and a better understanding of the disease.[9]

1.2.1 Breast Cancer Epidemiology

Female breast cancer has now surpassed lung cancer as the leading cause of global cancer incidence in 2020, with an estimated 2.3 million new cases, representing 11.7% of all cancer cases. It is the fifth leading cause of cancer mortality worldwide, with 685,000 deaths. Among women, breast cancer accounts for 1 in 4 cancer cases and for 1 in 6 cancer deaths, ranking first for

incidence in the vast majority of countries (159 of 185 countries) and for mortality in 110 countries.[10]

Worldwide, there will be about 2.1 million newly diagnosed female breast cancer cases in 2018, accounting for almost 1 in 4 cancer cases among women. The disease is the most frequently diagnosed cancer in the vast majority of the countries (154 of 185) and is also the leading cause of cancer death in over 100 countries; the main exceptions are Australia/New Zealand, Northern Europe, Northern America (where it is preceded by lung cancer), and many countries in Sub-Saharan Africa (because of elevated cervical cancer rates). Breast cancer incidence rates are highest in Australia/New Zealand, Northern Europe (eg, the United Kingdom, Sweden, Finland, and Denmark), Western Europe (Belgium [with the highest global rates], the Netherlands, and France), Southern Europe (Italy), and Northern America.[11]

An estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). A slight majority of cases occur in women in less developed regions. Breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths) and while it is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total), it is now the second cause of cancer death in more developed regions (198,000 deaths, 15.4%) after lung cancer (Table 6). The range in mortality rates between world regions is less than that for incidence because of the more favorable survival from breast cancer in (high-incidence) developed regions.[12]

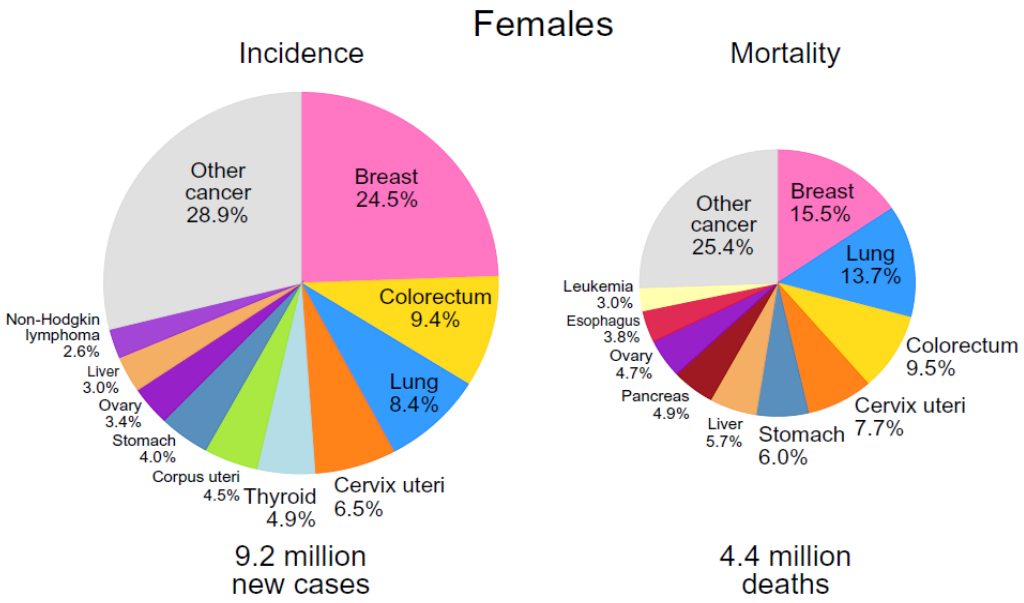


FIGURE 3: EPIDEMIOLOGICAL SURVEY OF BREAST CANCER IN FEMALES 2020[10]

Although hereditary and genetic factors, including a personal or family history of breast or ovarian cancer and inherited mutations (in *BRCA1*, *BRCA2*, and other breast cancer susceptibility genes), account for 5% to 10% of breast cancer cases, studies of migrants have shown that nonhereditary factors are the major drivers of the observed international and interethnic differences in incidence.

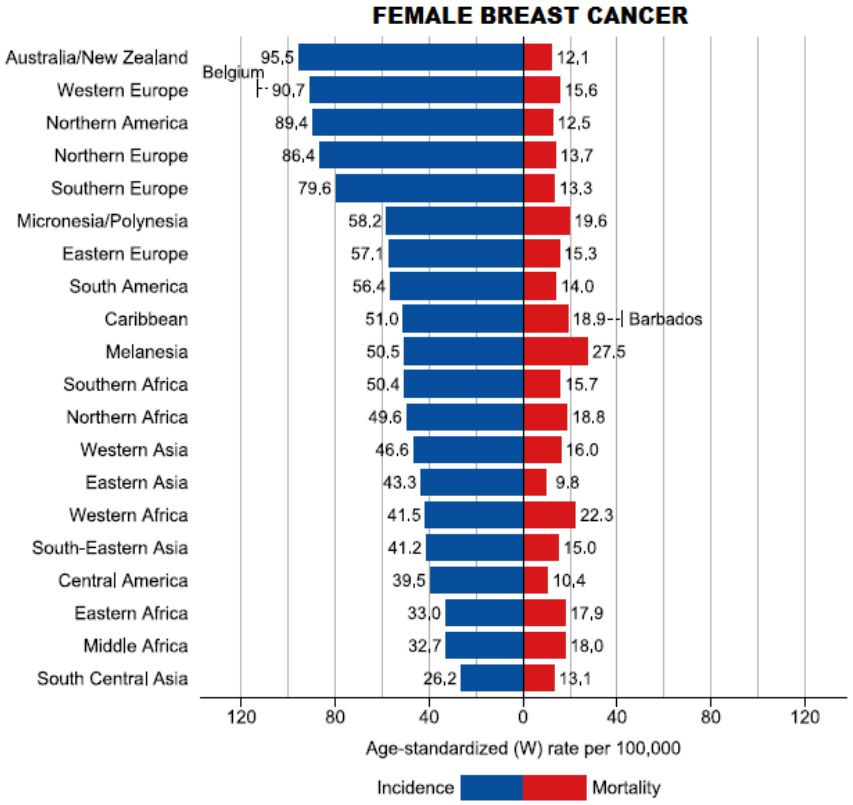


FIGURE 4: EPIDEMIOLOGICAL SURVEY OF BREAST CANCER 2020 [10]

1.3 Pathophysiology of Breast cancer

Breast cancers can start from different parts of the breast. Most breast cancers begin in the ducts that carry milk to the nipple (ductal cancers) some start in the glands that make breast milk (lobular cancers). There are also other types of breast cancer that are less common like phyllodes tumor and angiosarcoma. A small number of cancers start in other tissues in the breast. These cancers are called sarcomas and lymphomas and are not really thought of as breast cancers. There are many different types of breast cancer and common ones include ductal carcinoma in situ (DCIS) and invasive carcinoma. Others, like phyllodes tumors and angiosarcoma are less common. Once a biopsy is done, breast cancer cells are tested for proteins called estrogen receptors, progesterone

receptors and HER2. The tumor cells are also closely looked at in the lab to find out what grade it is. The specific proteins found and the tumor grade can help decide treatment options.[13]

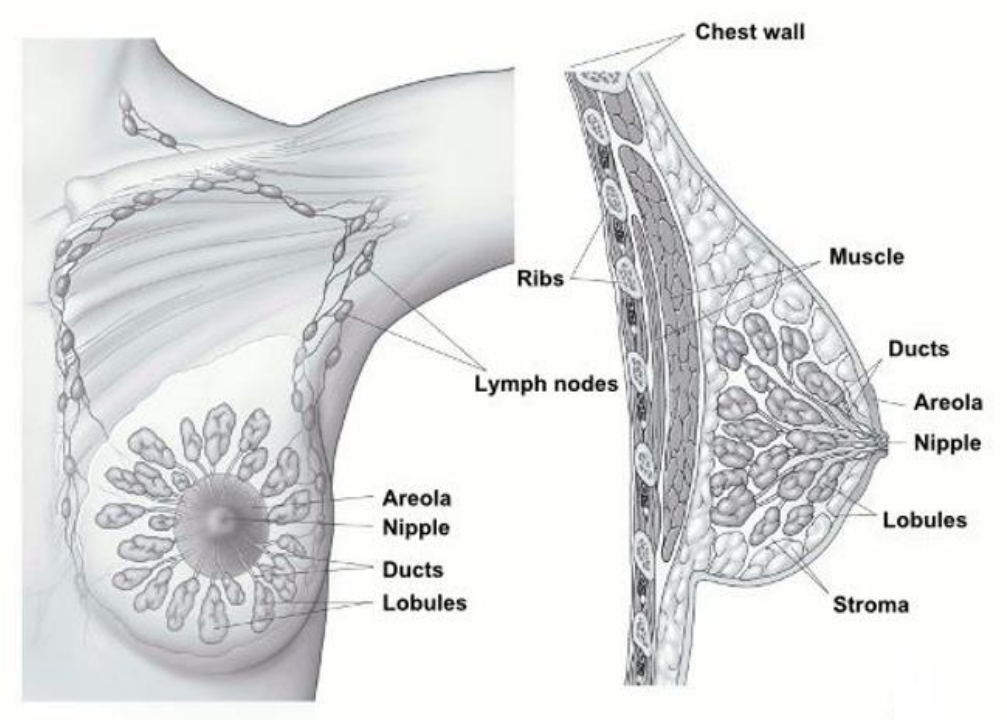


FIGURE 5: ANATOMY OF BREAST

Breast cancer can spread when the cancer cells get into the blood or lymph system and are carried to other parts of the body. The lymph system is a network of lymph (or lymphatic) vessels found throughout the body that connects lymph nodes (small bean-shaped collections of immune system cells). The clear fluid inside the lymph vessels, called lymph, contains tissue by-products and waste material, as well as immune system cells. The lymph vessels carry lymph fluid away from the breast. In the case of breast cancer, cancer cells can enter those lymph vessels and start to grow in lymph nodes. Most of the lymph vessels of the breast drain into:

- Lymph nodes under the arm (axillary nodes)
- Lymph nodes around the collar bone (supraclavicular [above the collar bone] and infraclavicular [below the collar bone] lymph nodes)
- Lymph nodes inside the chest near the breast bone (internal mammary lymph nodes)
- If cancer cells have spread to the lymph nodes, there is a higher chance that the cells could have traveled through the lymph system and spread (metastasized) to other parts of the

body. The more lymph nodes with breast cancer cells, the more likely it is that the cancer may be found in other organs.

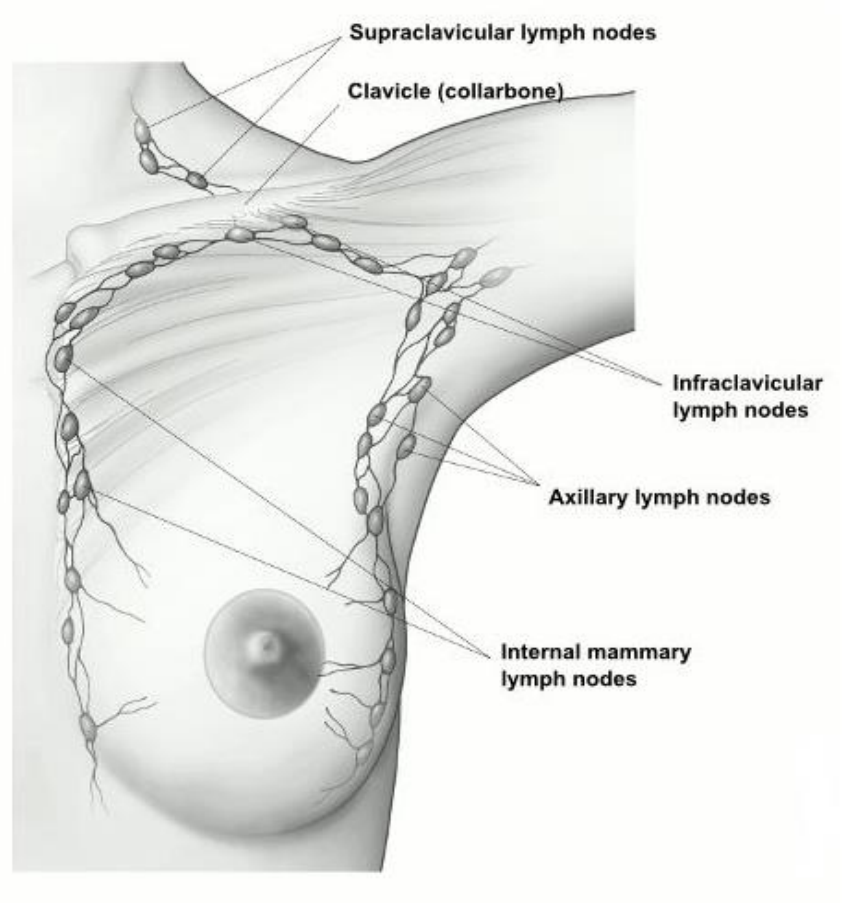


FIGURE 6: LYMPH NODES RELATION TO BREAST

1.3.1 Pathways involved in Breast Cancer

Breast cancers (BC) express the estrogen receptor (ER), progesterone receptor (PgR), or both, and such tumors are considered hormone receptor-positive (HR1). In addition to testing for the presence of ER and PgR, testing for Human Epidermal Growth Receptor 2 (HER2) protein overexpression and/or HER2 gene amplification is also performed. Molecular profiling has uncovered intrinsic subtypes in BC, including luminal A, luminal B, HER2-enriched, basal-like, and normal-like, which are associated with specific morphological and molecular features of BC. Over the last decade we have also improved our understanding of intracellular signaling pathways and the cancer cell cycle.

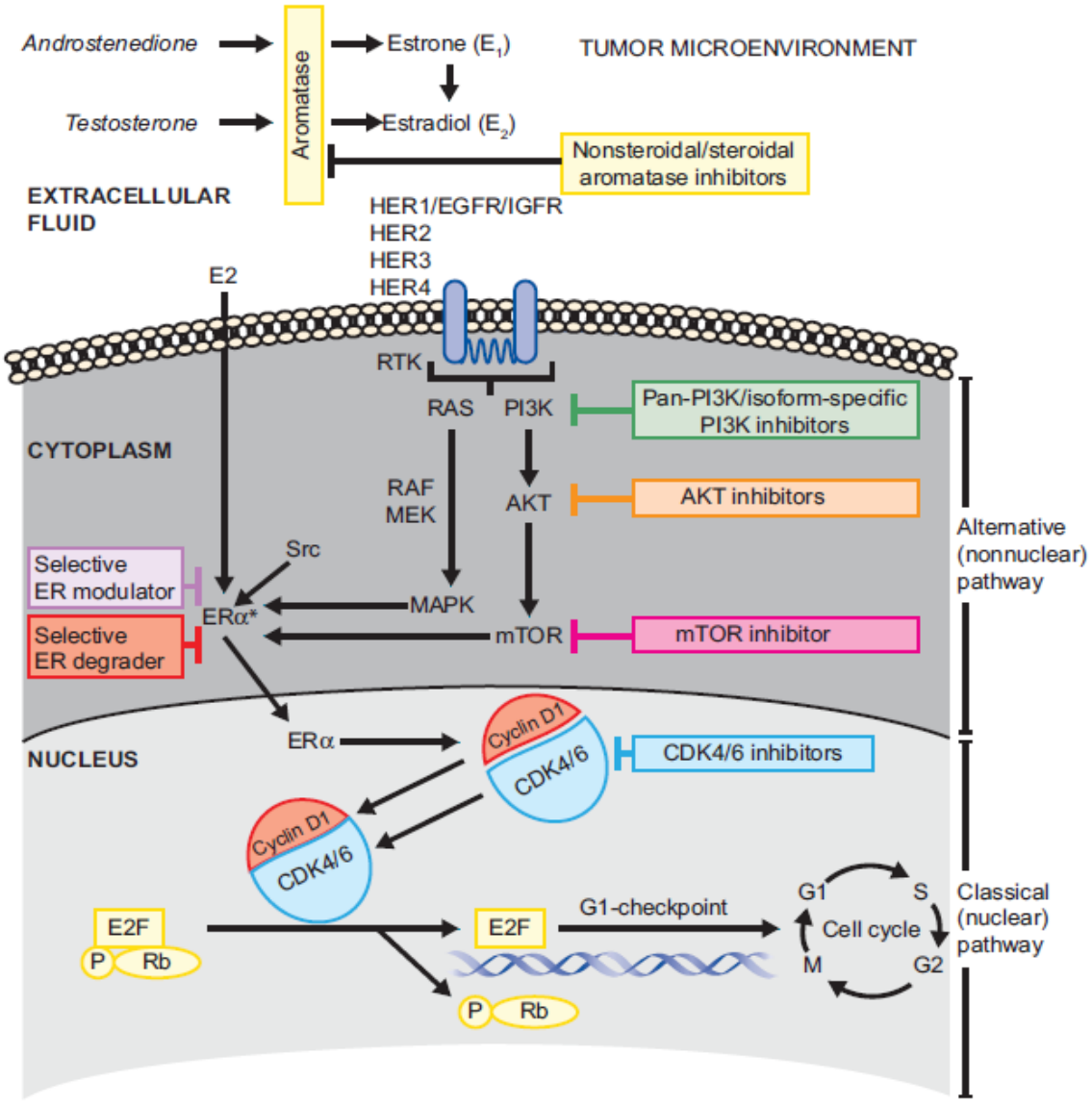


Figure 7: Pathways in Breast Cancer - Critical nuclear (classical) and nonnuclear (alternative) signaling pathways implicated in endocrine resistance and targets for drugs in development. Upon ligand binding, the estrogen-ER complex dimerizes and interacts with coregulator proteins and specific sequences of DNA and the estrogen response elements to promote the transcription of a wide range of genes that participate in the regulation of the cell cycle, DNA replication, cellular differentiation, apoptosis, and angiogenesis.

Abbreviations: AKT, akt murine thymoma viral oncogene; CDK4/6, cyclin-dependent kinase 4/6; E2, estradiol; E2F, E2F transcription factors; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERa, estrogen receptor-a; HER1, human epidermal growth receptor 1; HER2, human epidermal growth receptor 2; HER3, human epidermal growth receptor 3; HER4, human epidermal growth receptor 4; IGFR, insulinlike growth factor receptor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated ERK-activating kinase; mTOR, mammalian target of rapamycin; P, phosphate; PI3K, phosphoinositide-3-kinase; RAF, rapidly accelerated fibrosarcoma; RAS, rat sarcoma; Rb, retinoblastoma protein; RTK, receptor tyrosine kinase; SERD, selective estrogen receptor downregulator; SERM, selective estrogen receptor modulator.

There are two functionally distinct ERs, ER-alpha (ERa, ESR1 gene) and ER-beta (ESR2 gene). ERa is a predominant endocrine regulatory protein in the breast and in estrogen-induced BC. Estrogen binds to the ER with high affinity and specificity and functions through two main types of pathways, the classical (or nuclear) pathway and the alternative (nonnuclear) pathway. Successful targeting of genes within these nuclear and nonnuclear pathways remains an important clinical goal. Along the classical pathway, the estrogen-ER complex dimerizes upon ligand binding and interacts with coregulator proteins and specific sequences of DNA called estrogen responsive elements. These interactions promote the transcription of a wide range of genes that participate in the regulation of the cell cycle, DNA replication, cellular differentiation, apoptosis, and angiogenesis.

The engagement of the ER with estrogen through nonnuclear pathways originates in the cytoplasm to trigger coregulator growth factor and G-protein coupled signaling. Coregulators in the nonnuclear pathways include receptors (e.g., insulin-like growth factor-1 receptor, fibroblast growth factor receptor [FGFR], HER2), and kinases (e.g., mitogen activated protein kinases, receptor tyrosine kinase, PI3K, AKT, mTOR, Src, and CDK). Because the ER can also be activated through ligand-independent mechanisms, multiple opportunities exist for crosstalk between the ER, growth factors, and protein kinases, which can activate or modulate ER activity.[14]

1.4 Types of breast cancer

There are several types of breast cancer, and they are classified into two main categories: “invasive” and “noninvasive,” or in situ.While invasive cancer has spread from the breast ducts or glands to other parts of the breast, noninvasive cancer has not spread from the original tissue.

These two categories are used to describe the most common types of breast cancer, which include:

- **Ductal carcinoma in situ:** Ductal carcinoma in situ (DCIS) is a noninvasive condition. With DCIS, the cancer cells are confined to the ducts in your breast and haven’t invaded the surrounding breast tissue.
- **Lobular carcinoma in situ:** Lobular carcinoma in situ (LCIS) is cancer that grows in the milk-producing glands of your breast. Like DCIS, the cancer cells haven’t invaded the surrounding tissue.

- **Invasive ductal carcinoma:** Invasive ductal carcinoma (IDC) is the most common type of breast cancer. This type of breast cancer begins in your breast's milk ducts and then invades nearby tissue in the breast. Once the breast cancer has spread to the tissue outside your milk ducts, it can begin to spread to other nearby organs and tissue.
- **Invasive lobular carcinoma:** Invasive lobular carcinoma (ILC) first develops in your breast's lobules and has invaded nearby tissue.

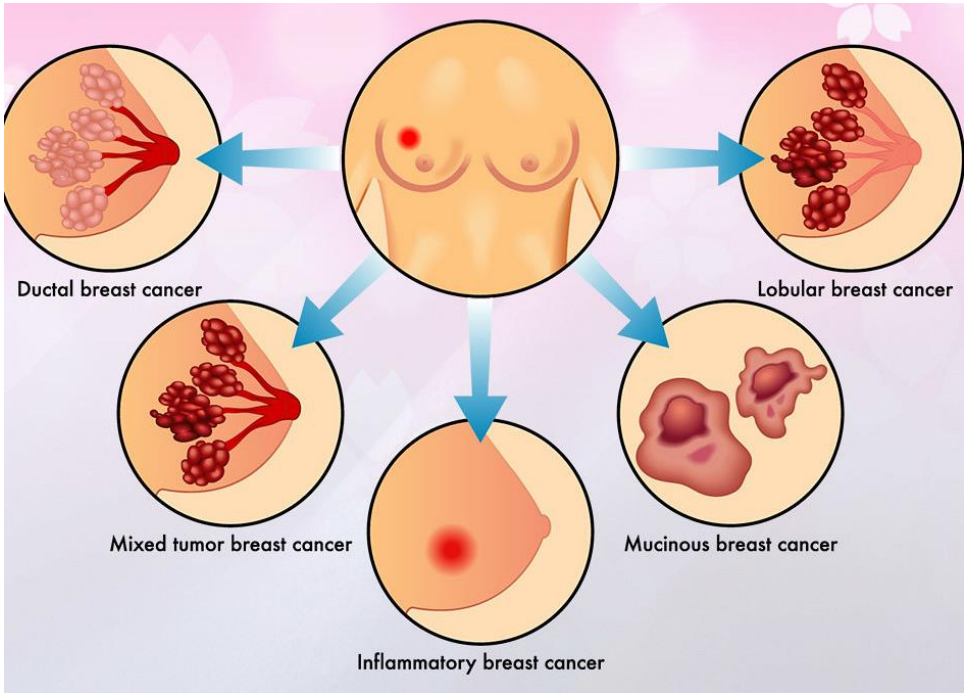


FIGURE 8: TYPES OF BREAST CANCER

Other, less common types of breast cancer include:

- **Paget disease of the nipple:** This type of breast cancer begins in the ducts of the nipple, but as it grows, it begins to affect the skin and areola of the nipple.
- **Phyllodes tumor:** This very rare type of breast cancer grows in the connective tissue of the breast. Most of these tumors are benign, but some are cancerous.
- **Angiosarcoma:** This is cancer that grows on the blood vessels or lymph vessels in the breast.

1.4.1 Inflammatory breast cancer

Inflammatory breast cancer (IBC) is a rare but aggressive type of breast cancer. IBC makes up only between 1 and 5 percent of all breast cancer cases. With this condition, cells block the lymph nodes near the breasts, so the lymph vessels in the breast can't properly drain. Instead of creating a tumor, IBC causes your breast to swell, look red, and feel very warm. A cancerous breast may appear pitted and thick, like an orange peel. IBC can be very aggressive and can progress quickly.

1.4.2 Metastatic breast cancer

Metastatic breast cancer is another name for stage 4 breast cancer. It's breast cancer that has spread from the breast to other parts of the body, such as bones, lungs, or liver. Tumor metastasis is a treacherous process that involves in several sequential steps, such as local invasion to escape from the surrounding tissues of the primary tumor, invading the blood or lymphatic vessels, survival in the circulation as circulating tumor cells (CTCs), escape of CTCs from the circulatory system, extravasation, adaptation to the microenvironment as disseminated tumor cells (DTCs), and transformation to metastasis initiating cells (MICs) to form a metastatic lesion.[15] Several similar mechanisms contribute to the early steps of metastasis, including local invasion, and survival in circulation. The epithelial-to-mesenchymal transition (EMT) is a critical step in initiating metastasis, incorporating the loss of epithelial markers, trans-differentiation into mesenchymal-like cells, and acquiring motile and invasive capacities.[16] [17]

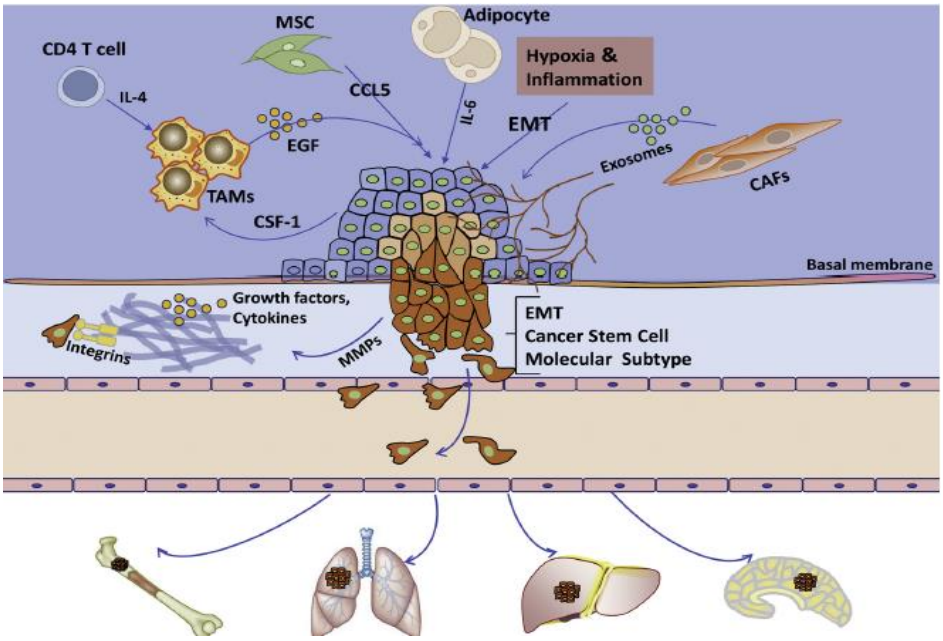


FIGURE 9: EARLY STAGE OF BREAST CANCER METASTASIS

1.4.3 Triple-negative breast cancer

Triple-negative breast cancer (TNBC), an aggressive phenotype associated with dismal survival, is considered as the most dangerous among all types of breast cancer.[18] TNBC cells show representative EMT associated with cancer progression. Triple-negative breast cancer is another rare disease type, affecting only about 10 to 15 percent of people with breast cancer, according to the American Cancer Society (ACS). To be diagnosed as triple-negative breast cancer, a tumor must have all three of the following characteristics:

- **It lacks estrogen receptors:** These are receptors on the cells that bind, or attach, to the hormone estrogen. If a tumor has estrogen receptors, estrogen can stimulate the cancer to grow.
- **It lacks progesterone receptors:** These receptors are cells that bind to the hormone progesterone. If a tumor has progesterone receptors, progesterone can stimulate the cancer to grow.
- **It doesn't have additional HER2 proteins on its surface:** HER2 is a protein that fuels breast cancer growth.

If a tumor meets these three criteria, it's labeled a triple-negative breast cancer. This type of breast cancer tends to grow and spread more quickly than other types of breast cancer. Triple-negative breast cancers are difficult to treat because hormonal therapy for breast cancer is not effective.

1.4.4 Stages of Breast cancer

Breast cancer can be divided into stages based on the size of the tumors and how much it has spread. Cancers that are large and/or have invaded nearby tissues or organs are at a higher stage than cancers that are small and/or still contained in the breast.[19]

To stage a breast cancer:

- If the cancer is invasive or noninvasive
- How large the tumor is
- Whether the lymph nodes are involved
- If the cancer has spread to nearby tissue or organs

Breast cancer has 5 main stages: stages 0 to 5.

Stage 0 breast cancer:

Stage 0 is DCIS. Cancer cells in DCIS remain confined to the ducts in the breast and have not spread into nearby tissue.

Stage 1 breast cancer

- **Stage 1A:** The primary tumor is 2 centimeters (cm) wide or less, and the lymph nodes are not affected.
- **Stage 1B:** Cancer is found in nearby lymph nodes, and either there is no tumor in the breast, or the tumor is smaller than 2 cm.

Stage 2 breast cancer:

- **Stage 2A:** The tumor is smaller than 2 cm and has spread to 1–3 nearby lymph nodes, or it's between 2 and 5 cm and hasn't spread to any lymph nodes.
- **Stage 2B:** The tumor is between 2 and 5 cm and has spread to 1–3 axillary (armpit) lymph nodes, or it's larger than 5 cm and hasn't spread to any lymph nodes.

Stage 3 breast cancer:

- **Stage 3A:** The cancer has spread to 4–9 axillary lymph nodes or has enlarged the internal mammary lymph nodes, and the primary tumor can be any size.

  Tumors are greater than 5 cm, and the cancer has spread to 1–3 axillary lymph nodes or any breastbone nodes.
- **Stage 3B:** A tumor has invaded the chest wall or skin and may or may not have invaded up to nine lymph nodes.
- **Stage 3C:** Cancer is found in 10 or more axillary lymph nodes, lymph nodes near the collarbone, or internal mammary nodes.

Stage 4 breast cancer:

Stage 4 breast cancer can have a tumor of any size, and its cancer cells have spread to nearby and distant lymph nodes as well as distant organs.

1.4.5 Signs and symptoms of breast cancer

In its early stages, breast cancer may not cause any symptoms. In many cases, a tumor may be too small to be felt, but an abnormality can still be seen on a mammogram. If a tumor can be felt, the first sign is usually a new lump in the breast that was not there before. However, not all lumps are cancer. Each type of breast cancer can cause a variety of symptoms. Many of these symptoms are similar, but some can be different. Symptoms for the most common breast cancers include:

- A breast lump or tissue thickening that feels different than surrounding tissue and has developed recently and Breast pain
- Red, pitted skin over your entire breast
- Swelling in all or part of your breast
- A nipple discharge other than breast milk
- Bloody discharge from your nipple
- Peeling, scaling, or flaking of skin on your nipple or breast

1.4.6 Screening methods for Breast cancer [20][21]

- **Breast exam:** Physician will check both of your breasts and lymph nodes in your armpit, feeling for any lumps or other abnormalities.
- **Mammogram:** A mammogram is an X-ray of the breast. Mammograms are commonly used to screen for breast cancer. If an abnormality is detected on a screening mammogram, your doctor may recommend a diagnostic mammogram to further evaluate that abnormality.[22]
- **Breast ultrasound:** Ultrasound uses sound waves to produce images of structures deep within the body. Ultrasound may be used to determine whether a new breast lump is a solid mass or a fluid-filled cyst.
- **Removing a sample of breast cells for testing (biopsy):** A biopsy is the only definitive way to make a diagnosis of breast cancer. During a biopsy, your doctor uses a specialized needle device guided by X-ray or another imaging test to extract a core of tissue from the suspicious area. Often, a small metal marker is left at the site within your breast so the area can be easily identified on future imaging tests. Biopsy samples are sent to a laboratory for analysis where experts determine whether the cells are cancerous. A biopsy sample is also

analyzed to determine the type of cells involved in the breast cancer, the aggressiveness (grade) of the cancer, and whether the cancer cells have hormone receptors or other receptors that may influence your treatment options.

- **Breast magnetic resonance imaging (MRI):** An MRI machine uses a magnet and radio waves to create pictures of the interior of your breast. Before a breast MRI, you receive an injection of dye. Unlike other types of imaging tests, an MRI doesn't use radiation to create the images.[23]

1.5 Treatments for Breast cancer

Breast cancer treatment options based on your type of breast cancer, its stage and grade, size, and whether the cancer cells are sensitive to hormones. Most women undergo surgery for breast cancer and many also receive additional treatment after surgery, such as chemotherapy, hormone therapy or radiation. Chemotherapy might also be used before surgery in certain situations. There are many options for breast cancer treatment, and you may feel overwhelmed as you make complex decisions about your treatment.[24]

1.5.1 Breast cancer surgery

Operations used to treat breast cancer include:

- **Removing the breast cancer (lumpectomy):** During a lumpectomy, which may be referred to as breast-conserving surgery or wide local excision, the surgeon removes the tumor and a small margin of surrounding healthy tissue. A lumpectomy may be recommended for removing smaller tumors. Some people with larger tumors may undergo chemotherapy before surgery to shrink a tumor and make it possible to remove completely with a lumpectomy procedure.
- **Removing the entire breast (mastectomy):** A mastectomy is an operation to remove all of your breast tissue. Most mastectomy procedures remove all of the breast tissue — the lobules, ducts, fatty tissue and some skin, including the nipple and areola (total or simple mastectomy). Newer surgical techniques may be an option in selected cases in order to improve the appearance of the breast. Skin-sparing mastectomy and nipple-sparing mastectomy are increasingly common operations for breast cancer.

- **Removing a limited number of lymph nodes (sentinel node biopsy):** To determine whether cancer has spread to your lymph nodes, your surgeon will discuss with you the role of removing the lymph nodes that are the first to receive the lymph drainage from your tumor. If no cancer is found in those lymph nodes, the chance of finding cancer in any of the remaining lymph nodes is small and no other nodes need to be removed.
- **Removing several lymph nodes (axillary lymph node dissection):** If cancer is found in the sentinel lymph nodes, your surgeon will discuss with you the role of removing additional lymph nodes in your armpit.
- **Removing both breasts:** Some women with cancer in one breast may choose to have their other (healthy) breast removed (contralateral prophylactic mastectomy) if they have a very increased risk of cancer in the other breast because of a genetic predisposition or strong family history. Most women with breast cancer in one breast will never develop cancer in the other breast.

1.5.2 Radiation therapy

Radiation therapy uses high-powered beams of energy, such as X-rays and protons, to kill cancer cells. Radiation therapy is typically done using a large machine that aims the energy beams at your body (external beam radiation). But radiation can also be done by placing radioactive material inside your body (brachytherapy).

External beam radiation of the whole breast is commonly used after a lumpectomy. Breast brachytherapy may be an option after a lumpectomy if you have a low risk of cancer recurrence.

Breast cancer radiation can last from three days to six weeks, depending on the treatment. A physician who uses radiation to treat cancer (radiation oncologist) determines which treatment is best for you based on your situation, your cancer type and the location of your tumor.

Side effects of radiation therapy include fatigue and a red, sunburn-like rash where the radiation is aimed. Breast tissue may also appear swollen or more firm. Rarely, more-serious problems may occur, such as damage to the heart or lungs or, very rarely, second cancers in the treated area.

1.5.3 Proton therapy

Standard radiation therapy for breast cancer uses x-rays, also called photon therapy, to kill cancer cells. Proton therapy is a type of external-beam radiation therapy that uses protons rather than x-rays. At high energy, protons can destroy cancer cells. Protons have different physical properties that may allow the radiation therapy to be more targeted than photon therapy and potentially reduce the radiation dose. The therapy may also reduce the amount of radiation that goes near the heart. Researchers are studying the benefits of proton therapy versus photon therapy in a national clinical trial. Currently, proton therapy is an experimental treatment and may not be widely available.

1.5.4 Chemotherapy

Chemotherapy uses drugs to destroy fast-growing cells, such as cancer cells. If your cancer has a high risk of returning or spreading to another part of your body, your doctor may recommend chemotherapy after surgery to decrease the chance that the cancer will recur. Chemotherapy is sometimes given before surgery in women with larger breast tumors. The goal is to shrink a tumor to a size that makes it easier to remove with surgery. Chemotherapy is also used in women whose cancer has already spread to other parts of the body. Chemotherapy may be recommended to try to control the cancer and decrease any symptoms the cancer is causing. Chemotherapy side effects depend on the drugs you receive. Common side effects include hair loss, nausea, vomiting, fatigue and an increased risk of developing an infection. Rare side effects can include premature menopause, infertility (if premenopausal), damage to the heart and kidneys, nerve damage, and, very rarely, blood cell cancer.[25]

1.5.5 Hormone therapy

Hormone therapy - perhaps more properly termed hormone-blocking therapy is used to treat breast cancers that are sensitive to hormones. Doctors refer to these cancers as estrogen receptor positive (ER positive) and progesterone receptor positive (PR positive) cancers. Hormone therapy can be used before or after surgery or other treatments to decrease the chance of your cancer returning. If the cancer has already spread, hormone therapy may shrink and control it.

Treatments that can be used in hormone therapy include:

- Medications that block hormones from attaching to cancer cells (selective estrogen receptor modulators)
- Medications that stop the body from making estrogen after menopause (aromatase inhibitors)
- Surgery or medications to stop hormone production in the ovaries

Hormone therapy side effects depend on your specific treatment, but may include hot flashes, night sweats and vaginal dryness. More serious side effects include a risk of bone thinning and blood clots.

1.5.6 Targeted therapy drugs

Targeted drug treatments attack specific abnormalities within cancer cells. As an example, several targeted therapy drugs focus on a protein that some breast cancer cells overproduce called human epidermal growth factor receptor 2 (HER2). The protein helps breast cancer cells grow and survive. By targeting cells that make too much HER2, the drugs can damage cancer cells while sparing healthy cells. Targeted therapy drugs that focus on other abnormalities within cancer cells are available. And targeted therapy is an active area of cancer research. Your cancer cells may be tested to see whether you might benefit from targeted therapy drugs. Some medications are used after surgery to reduce the risk that the cancer will return. Others are used in cases of advanced breast cancer to slow the growth of the tumor.[26]

1.5.7 Immunotherapy

Immunotherapy uses your immune system to fight cancer. Your body's disease-fighting immune system may not attack your cancer because the cancer cells produce proteins that blind the immune system cells. Immunotherapy works by interfering with that process. Immunotherapy might be an option if you have triple-negative breast cancer, which means that the cancer cells don't have receptors for estrogen, progesterone or HER2. For triple-negative breast cancer, immunotherapy is combined with chemotherapy to treat advanced cancer that's spread to other parts of the body.[27]

1.5.8 Supportive (palliative) care

Palliative care is specialized medical care that focuses on providing relief from pain and other symptoms of a serious illness. Palliative care specialists work with you, your family and your other

doctors to provide an extra layer of support that complements your ongoing care. Palliative care can be used while undergoing other aggressive treatments, such as surgery, chemotherapy or radiation therapy. When palliative care is used along with all of the other appropriate treatments, people with cancer may feel better and live longer. Palliative care is provided by a team of doctors, nurses and other specially trained professionals. Palliative care teams aim to improve the quality of life for people with cancer and their families. This form of care is offered alongside curative or other treatments you may be receiving.

1.5.9 Limitation of Current therapy

A major advantage of chemotherapy is its ability to travel throughout the body and attack widespread cancers, whereas surgery and radiation are confined to treat one area. The disadvantages of chemotherapy are the toxic side effects, the development of resistance to the chemical agents, and the need for other forms of treatment, in combination with chemotherapy, in order to cure the patient. These disadvantages are why molecular based treatments are becoming so valuable. Most molecular based treatments are designed specifically to destroy only cancer cells. Since molecular based treatments are specific they are not associated with the same toxic side effects as chemotherapy. The understanding of the molecular basis of cancer would allow us to improve molecular treatments because unfortunately they cannot perform efficiently without including other forms of treatments.

1.6 Hallmarks of Breast cancer

1. Sustaining proliferative signaling
2. Evading growth suppressors
3. Resisting cell death
4. Enabling replicative immortality
5. Inducing angiogenesis
6. Activating invasion and metastasis
7. Reprogramming of energy metabolism
8. Evading immune destruction
9. Genome instability
10. Tumor-promoting inflammation[28]

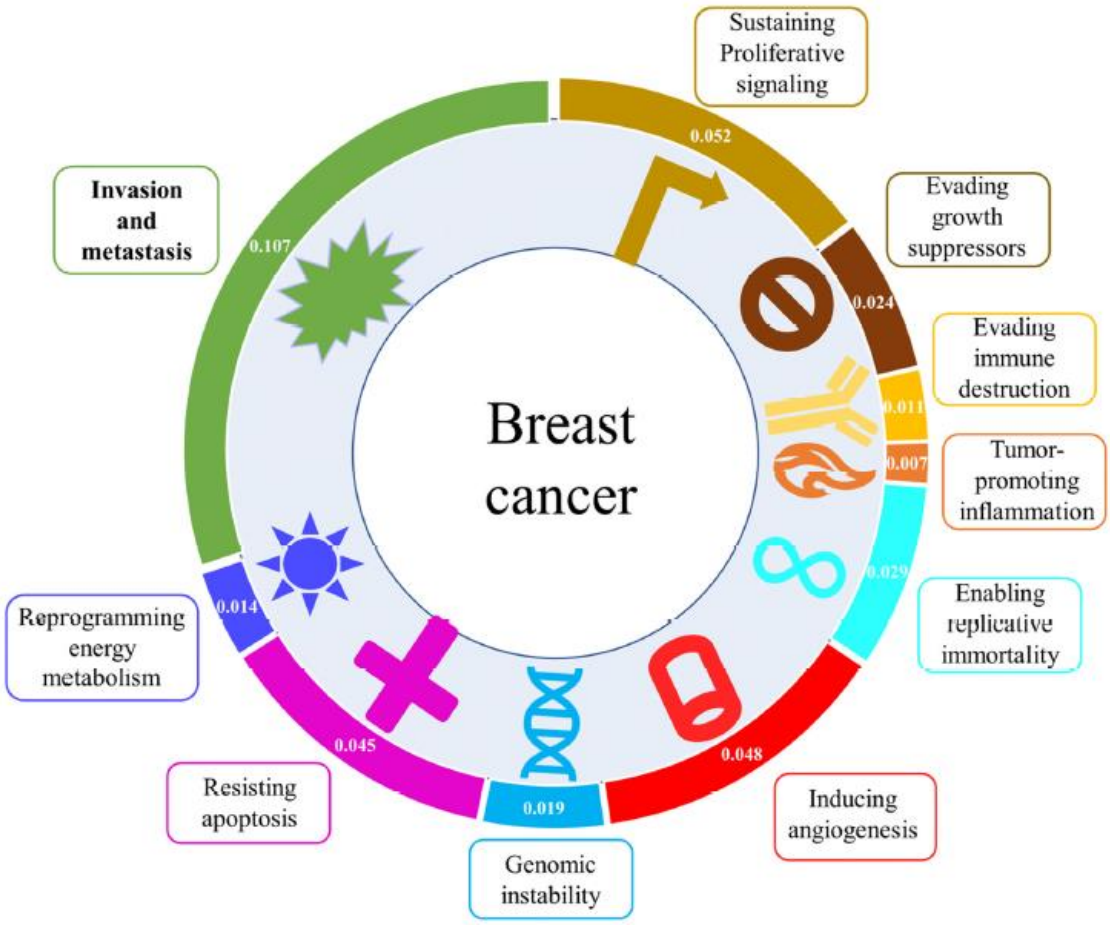


Figure 10: Hallmarks of Breast cancer

1.6.1 Target identification in Breast cancer

TABLE 1: Targets in Breast cancer

S.NO	TARGETS	FUNCTION
1.	MDR1 (Multi Drug Resistance protein-1)	P-gp efflux mechanism
2.	ABCB1 (ATP Binding Cassette sub-family B member-1)	MDR gene of tumor cells
3.	AP2 (Adaptor Protein-2)	Clathrin mediated endocytosis
4.	Cav-1 (Caveolin-1 or Cavin protein)	Caveolin mediated endocytosis
5.	Transferrin	Carrier protein

1.6.2 MDR1 (Multi Drug Resistance protein-1)

Multidrug resistance (MDR) is another refractory outcome of chemotherapy and is defined as the resistance of cancer cells to multiple chemotherapeutic drugs with different structures and mechanisms of action. MDR is a major cause of chemotherapy failure and responsible for increasing cancer-related mortality. MDR1 belong to the P-gp gene subfamily is mostly seen in

humans. Where MDR1 (P-gp) is widely seen all over the body and efflux a wide range of drugs over the plasma membrane.[29][30]

P-gp has a leading aspect in reducing the bioavailability and distribution of the drugs, where P-gp is over-expressed in intestinal region which act as a substrate to P-gp and reduces its absorption pathway. Hence the therapeutic level of the drugs and the bioavailability of the drugs are not accomplished. On the other hand if the P-gp expression is abscessed then the concentration of the drug in the plasma will reach to supra-therapeutic concentration leading to toxicity related issues. Over-expression of the P-gp is the foremost reason behind the failure of the chemotherapy and other treatment strategies. The compounds transported by P-gp are considered as substrates, while the compounds that prohibit the role of P-gp are considered as inhibitors.[31]

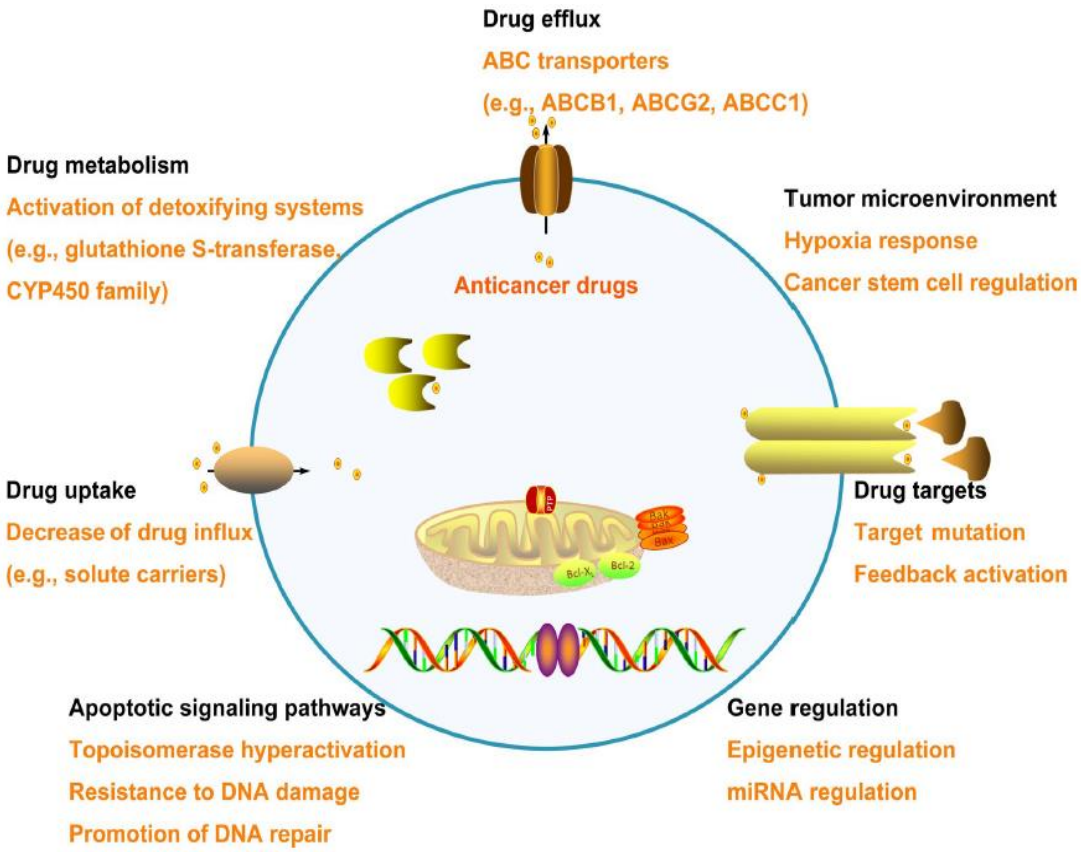


FIGURE 11: Mechanisms of multidrug resistance (MDR) - The main mechanism of MDR is overexpressing ATP-binding cassette (ABC) transporters to increase drug efflux, resulting in a decrease in intracellular drug concentration. Other mechanisms of MDR are reducing drug uptake by influx transporters, boosting drug metabolism, blocking apoptotic signaling pathways and mutation in drug targets or feedback activation of other targets and signaling pathways and change of tumor microenvironment.

1.6.3 ABCB1 (ATP Binding Cassette sub-family B member-1)

P-gp is an efflux protein system associated with the ATP binding cassette (ABC) sub-family B membrane. The ABC superfamily contains 49 different types of transporters and can be classified into seven subfamilies from ABC-A to ABC-G based on sequence similarities and structural organization.[32] Among them, P-glycoprotein (P-gp/ABCB1), multidrug-resistant protein 1 (MRP1/ABCC1), breast cancer resistant protein (BCRP/ ABCG2/ABCP), and multidrug-resistant protein 10 (ABCC10/MRP7) transporters frequently drive chemosensitive cancers to MDR. Human ABCB1 was the first identified ABC transporter. ABC gene indicates the leading family of TM (trans-membrane) protein, originated mainly in the intercellular membrane or the plasma membrane. By using the energy from the ATP the transport mechanism across the cell membrane is initiated.[33] Overexpression of ABCB1 contributes to resistance against a wide variety of chemotherapeutic drugs. ABCC1 also leads to resistance to a wide range of anticancer drugs, and extensive evidence indicates that resistance of cancer cells. ABC transporters are recognized as chief culprits in the development of MDR. Therapies continue to be developed with the goal of blocking or inactivating ABC transporters to increase the concentration of anticancer drugs within cells.[34]

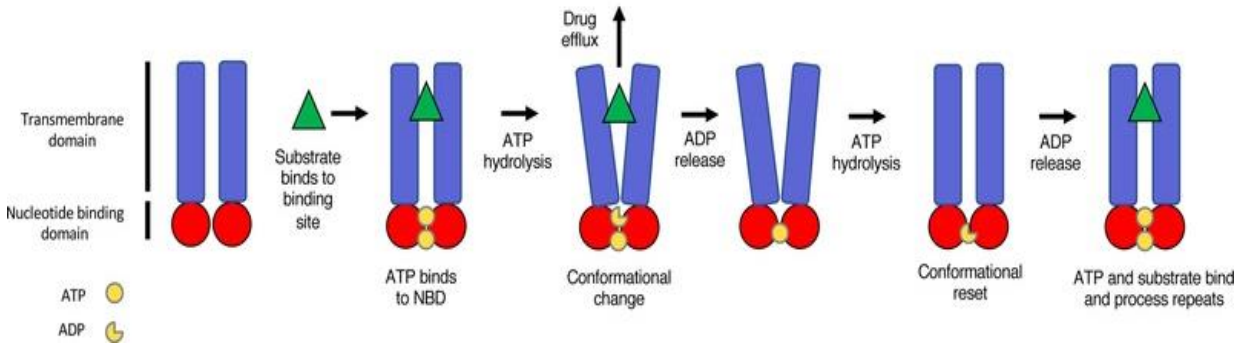


FIGURE 12: Mechanism of ABCB1 (ATP Binding Cassette sub-family B member-1)

1.6.4 AP2 (Adaptor Protein-2) - Clathrin mediated endocytosis

Clathrin was one of the first endocytic coat proteins to be discovered as a major component of 'bristled vesicles'. Clathrin functions as a trimer of heterodimers, each unit consisting of one heavy and one light chain forming a triskelion. These triskelia can assemble into a lattice-like structure around the vesicles. Adaptor proteins function to link up specific cargo with the Clathrin coat.

Clathrin mediated endocytosis is a cargo-induced process, it is now known that Clathrin coats can spontaneously assemble at the PM (plasma membrane) and are stabilized by interactions with cargo.[35] One of the best-characterized vesicular carriers are the Clathrin-coated vesicles (CCV). The major structural unit of the Clathrin coat is the triskelion, which is facilitated by the presence of the Clathrin heterotetrameric adaptor proteins (APs). The APs are positioned between the Clathrin lattice and the vesicle membrane and are proposed to link cargo selection to Clathrin coated pit formation. CCVs associated with AP-1 are involved in lysosome biogenesis, while endocytosis is mediated by CCVs, associated with AP-2, that form at the plasma membrane.[36]

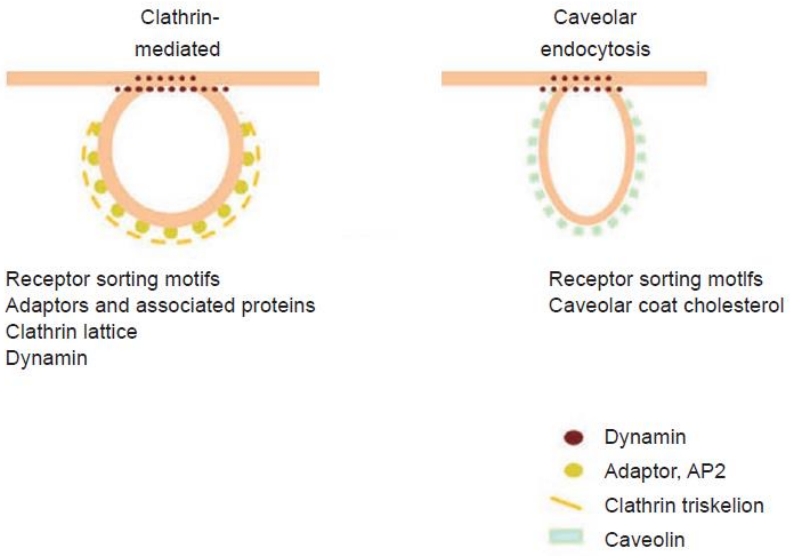


FIGURE 13: Clathrin and Caveolin mediated endocytosis

1.6.5 Cav-1 (Caveolin-1 or Cavin protein) - Caveolin mediated endocytosis

Like Clathrin, another membrane coat at the cell surface is Caveolin. Caveolae were originally described as flask-shaped structures. Caveolae are formed by assembly of caveolins, integral membrane proteins that bind directly to membrane cholesterol.[37] There are three subtypes of Caveolin proteins, caveolin-1, caveolin- 2, and caveolin-3, the former two being responsible for Caveolae formation in non-muscle cells and latter in muscle cells. Caveolins have a predicted hydrophobic stretch of potential hairpin structure composed of α-helices.[38] After the concentration of endocytic receptors into Clathrin-coated pits, CCVs must be formed and released from the plasma membrane. The latter step of pinching off from the plasma membrane was

proposed to be mediated by the large GTPase dynamin, working as a mechanochemical enzyme or pinchase. Dynamin more likely plays a regulatory role in this process. Dynamin has also been implicated in internalization mediated by Caveolae, flask-shaped structures that are morphologically, biochemically, and functionally distinguishable from Clathrin-coated pits.[39]

TABLE 2: Summary of Endocytic mechanisms

S.NO	Endocytic mechanisms	Clathrin-mediated	Caveolin-mediated
1.	Morphology and Size	Vesicular Flask, 150-200 nm	Flask-shaped, ~120 nm
2.	Implicated coat	Clathrin	Caveolin
3.	Dynamin - dependence	Yes	Yes
4.	Small GTPase involved	Rab5	Not well established
5.	Other proteins associated	AP2, Eps15, Epsin Amphiphysin	PTRF, src, SDPR Actin
6.	Cargo (examples)	Transferrin receptor, EGF (at low concentrations) GPCRs, RTKs Ebola, SARS, and some other viruses	Albumin, AMF Cholera toxin, Tetanus toxin, SV40 Polyoma viruses

1.6.6 Transferrin (Carrier protein)

The transferrins are a family of iron-binding proteins, which has a high affinity to ferric iron. Transferrin is a blood-plasma glycoprotein, which plays a central role in iron metabolism and is responsible for ferric-ion delivery. Iron is a vital element for several metabolic pathways and physiological processes. The maintenance of iron homeostasis is essential as a change either decrease or excess pose harmful to the human body. Transferrin functions as the most critical ferric pool in the body. It transports iron through the blood to various tissues such as the liver, spleen and bone marrow. It is an essential biochemical marker of body iron status.[40] The process of offloading iron-bound transferrin begins with transferrin binding to its cell surface transferrin receptor. It starts with the formation of Clathrin-coated pits and internalization of the vesicle into the cytoplasm. Clathrin/receptor-mediated endocytosis mediates the uptake of iron by transferrin receptors. The coated vesicle loses its Clathrin coat due to a reduction in pH, which encourages the release of iron from its binding site and endocytosed into a cell. After the dissociation of iron, transferrin is called apotransferrin. Apotransferrin remains bound to its receptor because it has a high affinity for its receptors at a reduced pH. At a neutral pH, apotransferrin dissociates from its receptor to enter the circulation, reload iron and repeat the cycle.[41]

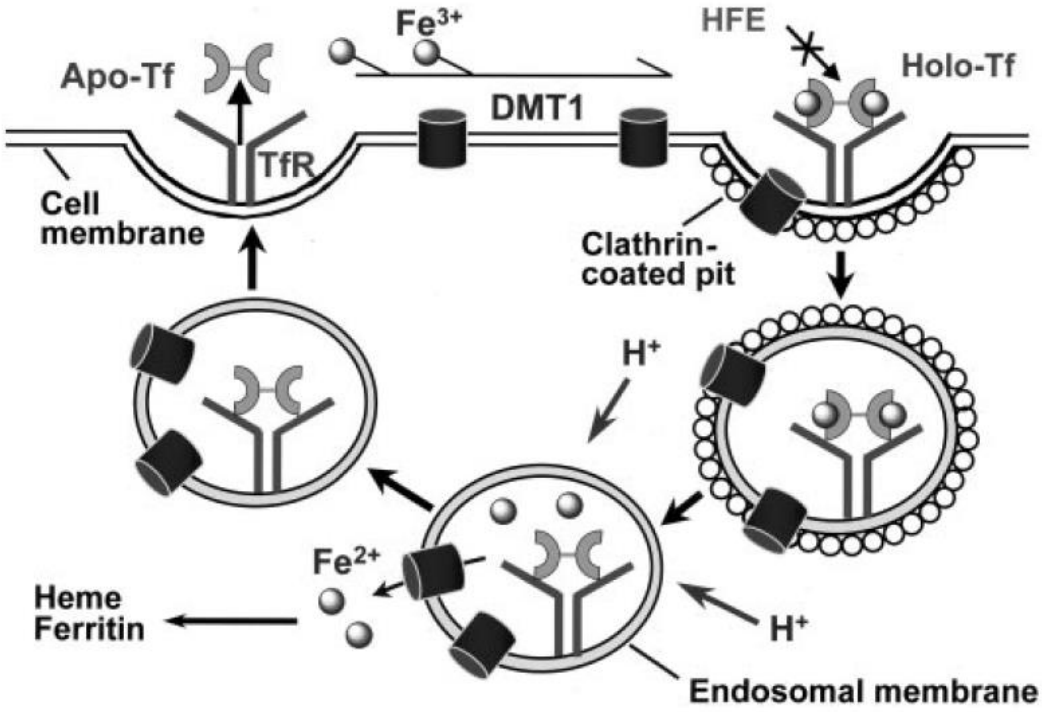


FIGURE 14: Mechanism of Transferrin receptor - The cycle of transferrin and transferrin receptor 1-mediated cellular iron uptake. Differic transferrin (holo-transferrin) binds to transferrin receptor 1 on the cell surface. The resulting complex is internalized and acidified through the action of a proton pump. Iron is subsequently released from transferrin and transported out of endosomes via the divalent metal transporter DMT1. HFE hemochromatosis protein (the protein mutated in hereditary hemochromatosis) appears to inhibit transferrin receptor 1 endocytosis, probably through binding to transferrin receptor 1. Apo-transferrin and transferrin receptor 1 both returned to the cell surface, where they dissociate at neutral pH, and both participate in another round cycle of iron uptake. Intracellular iron is either incorporated into heme or stored in ferritin.

1.7 PLANT PROFILE OF *ANNONA MURICATA*

Annona muricata, commonly called soursop (also known as graviola or guanabana), belongs to Annonaceae family comprising approximately 130 genera and 2300 species. It is a small evergreen tropical tree that is native to Central and tropical South America. In the wild, it is a slender tree that will grow to 15-20' tall often with down-curved branching. It grows much smaller in containers. The name soursop is in reference to its large edible fruits which have a slightly acidic or sour taste. This tropical plant is not reliably winter hardy to most of southern Florida, but may be grown in the Florida Keys and Puerto Rico. In greenhouses, it typically flowers in summer producing fruits in fall. Each flower (to 2" long) appears singly on a woody stalk almost anywhere on the tree (twigs, branches or trunk). Each flower has three yellowish-green outer petals and three dull yellow inner petals. Flowers give way to rough-skinned, prickly-textured, oblong fruit (each fruit from 6-12" long to 4-6" wide with weight to 8-15 pounds). Fruit pulp is used in ice cream, sherbets or juices. Leathery, egg-shaped, malodorous (when bruised), oblong to oval leaves (to 6" long) are glossy green above. Plants have been used for various medicinal purposes.[42]

FIGURE 15: (15a) *Annona muricata* tree; (15b) *Annona muricata* leaves; (15c) *Annona muricata* fruit, and (15d) *Annona muricata* flower

TABLE 3: Plant Description

S.NO	MORPHOLOGY	DESCRIPTION
1.	Tamil Name	Mullu-sitha-pazham
2.	Common Name	soursop
3.	Type	Tree
4.	Family	Annonaceae
5.	Native Range	Central and tropical South America
6.	Zone	10 to 11
7.	Height	15.00 to 20.00 feet
8.	Spread	8.00 to 12.00 feet
9.	Bloom Time	June to August
10.	Bloom Description	Yellow-green
11.	Sun	Full sun to part shade
12.	Water	Medium
13.	Maintenance	Low
14.	Flower	Showy
15.	Leaf	Evergreen
16.	Fruit	Showy, Edible

IDENTIFICATION OF LIGANDS

1.7.1 Phytoconstituents

A. muricata has been found to contain 212 bioactive compounds that include acetogenins (AGEs), alkaloids, and phenolic acids. AGEs are least investigated, polyether compounds found exclusively in the plants belonging to *Annonaceae* family. They comprise an aliphatic chain containing 32–34 carbons, attached to a terminal lactone or butenolide ring. Epoxide, hydroxyl, ketone, tetrahydrofuran (THF), and tetrahydropyran groups may also be featured in AGEs. In the *A. muricata* species alone, more than 120 AGEs have been reported. A THF ring is found in most of the AGEs constituting *A. muricata*. Biomedical properties and toxicity of *A. muricata* are attributed to the presence of AGEs.[43]

1.7.2 *Annonaceous* Acetogenins

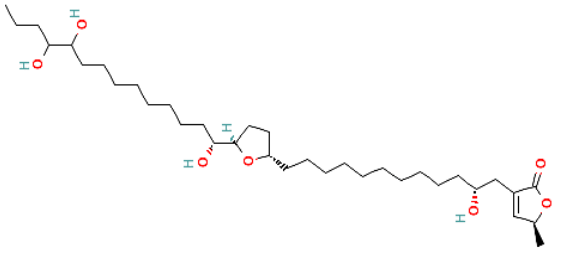
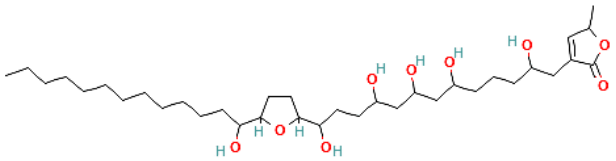
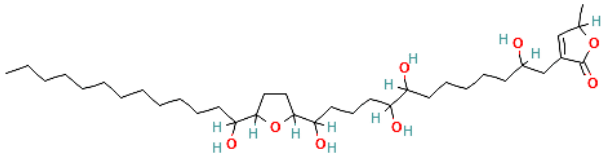
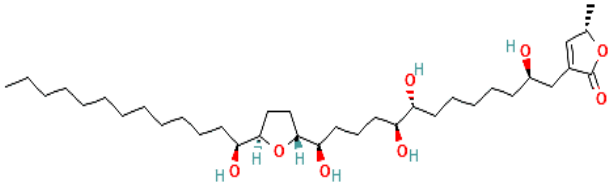
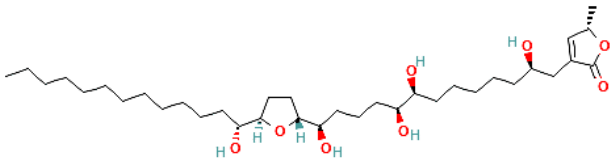
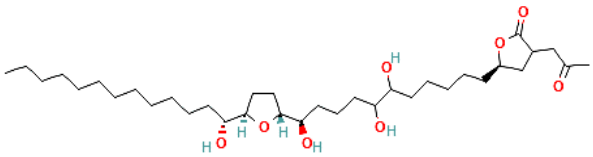
AGEs are a unique class of C-35/C37 secondary metabolites derived from long chain (C-32/C34) Fatty acids in the polyketide pathway. They are usually characterized by a combination of fatty acids with a 2-propanol unit at C-2 that forms a methyl-substituted α, β-unsaturated γ-lactone. More than 100 AGEs have been identified from different parts of the plants in the Annonaceae family. Due to the special structures and extensive biological activities, AGEs have attracted

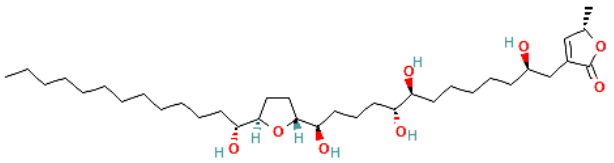
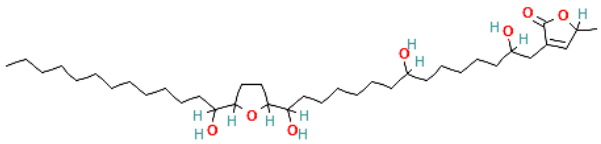
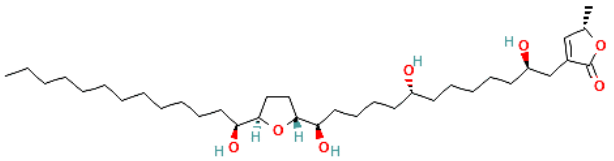
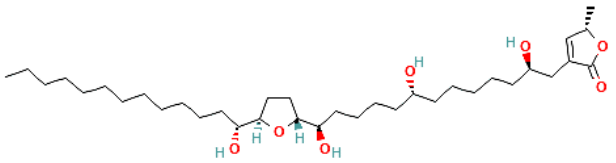
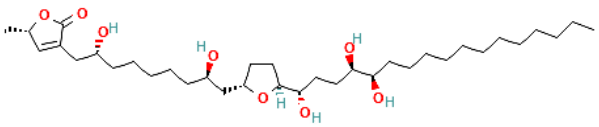
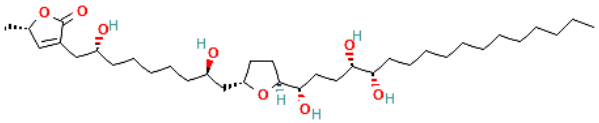
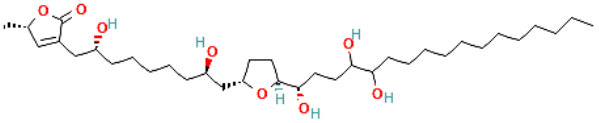
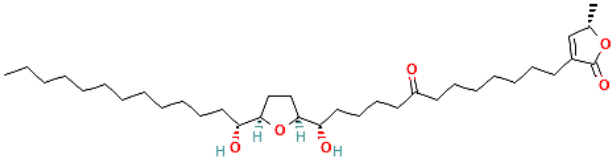
significant scientific interest in recent years. Various biological activities have been reported for AGEs, including antimalarial, antiparasitic and pesticidal activities. However, the biological activities of AGEs are primarily characterized with toxicity against cancer cells and inhibitory effects against the mitochondrial complex I (mitochondrial NADH: ubiquinone oxidoreductase). Phytochemical investigations and biological studies on different parts of the *A. muricata* plant resulted in the identification of a wide array of AGE compounds.[44]

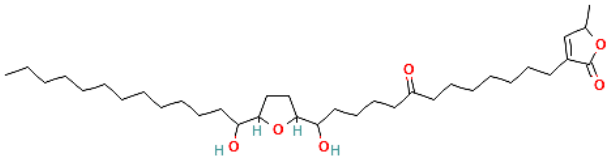
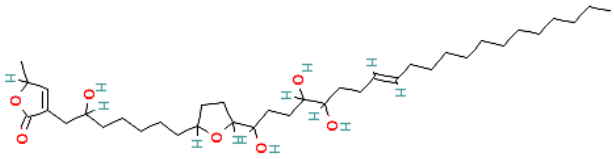
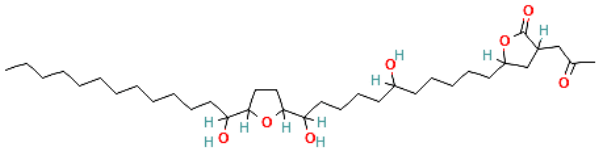
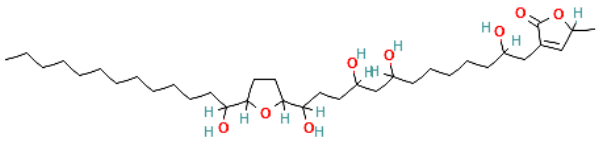
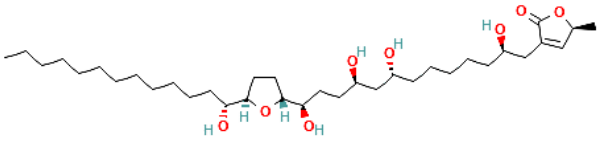
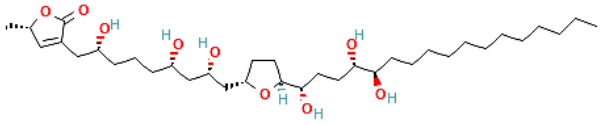
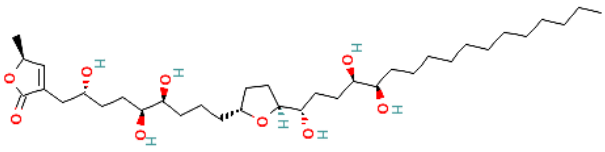
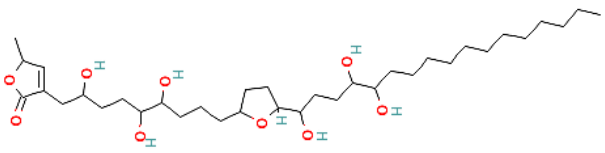
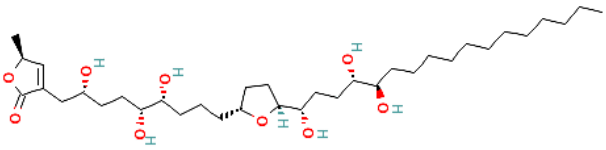
1.7.3 Rational uses of *Annona muricata* (Biomedical properties of Acetogenin)

Annonaceous acetogenins, exclusive to the *Annonaceae* family of the plant kingdom, have been the principal bioactive compounds. Annonaceous acetogenins have been the key phytochemical agents responsible for the anticancer activity of the plant. The above class of phytochemicals was found to be more potent as compared to the prescribed chemotherapeutic drugs.[45] The cytotoxicity observed in tumor cells is mainly attributed to the physiological activities of these AGEs, leading to the inhibition of mitochondrial complex and therefore resulting in apoptosis.[46] Nonetheless, ethanol extracts of the *A. muricata* leaves were reported to down regulate epidermal growth factor receptor, an oncogene overexpressed in breast cancer.[47] *A. muricata* fruit extracts for selective cytotoxicity, inhibiting the growth of MDA-MB-468 cells, having a greater Epidermal growth factor receptor (EGFR) overexpression, and not the normal breast epithelial MCF-10 cells. In addition, various studies ascertain potent tumoricidal properties of *A. muricata* phytochemicals against hepatoma, pancreatic adenocarcinoma, and prostate cancer.[48][49]

TABLE 4: Phytoconstituents of Acetogenin (AGE) derivatives [50]

S. no	Compounds	Structure	Molecular formula
1.	Annocatalin		$C_{35}H_{64}O_7$
2.	Annohexocin		$C_{35}H_{64}O_9$
3.	Annomuricin A		$C_{35}H_{64}O_8$
4.	Annomuricin B		$C_{35}H_{64}O_8$
5.	Annomuricin C		$C_{35}H_{64}O_8$
6.	Annomuricin-D-One		$C_{35}H_{64}O_8$

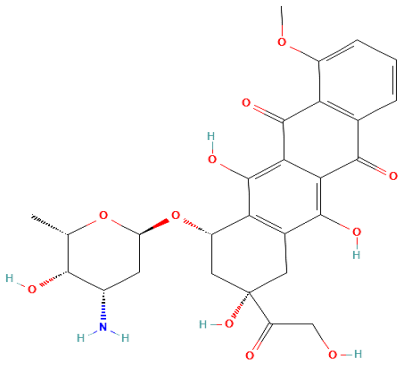
7.	Annomuricin E		$C_{35}H_{64}O_8$
8.	Annomutacin		$C_{37}H_{68}O_7$
9.	Annonacin A		$C_{35}H_{64}O_7$
10.	Annonacin		$C_{35}H_{64}O_7$
11.	Annopentocin A		$C_{35}H_{64}O_8$
12.	Annopentocin B		$C_{35}H_{64}O_8$
13.	Annopentocin C		$C_{35}H_{64}O_8$
14.	Cis-Corossolone		$C_{35}H_{62}O_6$

15.	Corossolone		$C_{35}H_{62}O_6$
16.	Gigantetronenin		$C_{37}H_{66}O_7$
17.	Iso-Annonacin		$C_{35}H_{64}O_7$
18.	Muricatocin B		$C_{35}H_{64}O_8$
19.	Muricatocin C		$C_{35}H_{64}O_8$
20.	Muricoreacin		$C_{35}H_{64}O_9$
21.	Murihexocin A		$C_{35}H_{64}O_9$
22.	Murihexocin C		$C_{35}H_{64}O_9$
23.	Murihexocin		$C_{35}H_{64}O_9$

1.8 DOXORUBICIN

Doxorubicin is an antineoplastic in the anthracycline class. Interaction with DNA in a variety of different ways including intercalation (squeezing between the base pairs), DNA strand breakage and inhibition with the enzyme topoisomerase II. Most of these compounds have been isolated from natural sources and antibiotics. The anthracyclines are among the most important antitumor drugs available. Doxorubicin is widely used for the treatment of several solid tumors while daunorubicin and idarubicin are used exclusively for the treatment of leukemia. Doxorubicin may also inhibit polymerase activity, affect regulation of gene expression, and produce free radical damage to DNA. Doxorubicin possesses an antitumor effect against a wide spectrum of tumors, either grafted or spontaneous. The anthracyclines are cell cycle-nonspecific. Terminal half-life is 20 - 48 hours. The distributive half-life is 5 minutes, which suggests that doxorubicin is rapidly taken up by tissue. Steady state volume of distribution = 809 to 1214 L/m2. Doxorubicin and its major metabolite, doxorubicinol, is 74-76% bound to plasma protein. The extent to binding is independent of plasma concentration up to 1.1 mcg/mL. Doxorubicin does not cross the blood brain barrier.[51][52]

TABLE 5: Description of Doxorubicin

S.NO	COMPOUND	STRUCTURE	MOLECULAR FORMULA
1.	Doxorubicin		$C_{27}H_{29}NO_{11}$

1.8.1 Drawback of Doxorubicin therapy

Doxorubicin has two major limiting factors, namely cardiotoxicity and emergence of multidrug resistance (MDR) to doxorubicin in cancerous cells. In most of the cancer patients, doxorubicin usually produces not so severe and quite easily manageable side effects like gastrointestinal disturbances (like nausea, vomiting, diarrhea, etc.), loss of hair (alopecia), oral ulcer (stomatitis), etc. However, cardiotoxicity remains to be one of the most serious side effects of doxorubicin. Reports have estimated that around 11% of the cancer patients receiving doxorubicin develops acute cardiotoxicity. Chronic cardiotoxicity is associated with progressive myocardial dysfunction, left ventricular (LV) systolic dysfunction, dilated cardiomyopathy and CHF. Besides heart, other organs namely kidney, liver, brain, etc. may also be affected adversely following doxorubicin therapy. Besides, adverse drug reaction (especially cardiotoxicity) emergence of MDR is one of the limiting factors of doxorubicin therapy.[53]

1.8.2 Current therapy of Doxorubicin

Doxorubicin is used to produce regression in disseminated neoplastic conditions like acute lymphoblastic leukemia, acute myeloblastic leukemia, Wilms' tumor, neuroblastoma, soft tissue and bone sarcomas, breast carcinoma, ovarian carcinoma, transitional cell bladder carcinoma, thyroid carcinoma, gastric carcinoma, Hodgkin's disease, malignant lymphoma and bronchogenic carcinoma in which the small cell histologic type is the most responsive compared to other cell types. Doxorubicin is also indicated for use as a component of adjuvant therapy in women with evidence of axillary lymph node involvement following resection of primary breast cancer.[54]

1.9 *In Silico* Drug Design

In silico is a term that means "computer aided". The phrase was coined in 1989 as an analogy to the Latin phrases *in vivo*, *in vitro*, and *in situ*.[55] So *in silico* drug design means rational design by which drugs are designed/discovered by using computational methods. Most of the drugs in the past were discovered by coincidence or trial and error method, or in other words, serendipity played an important role in finding new drugs. The use of computers and computational methods permeates all aspects of drug discovery today and forms the core of structure-based drug design. High-performance computing, data management software and internet are facilitating the access of huge amount of data generated and transforming the massive complex biological data into

workable knowledge in modern day drug discovery process. The use of complementary experimental and informatics techniques increases the chance of success in many stages of the discovery process, from the identification of novel targets and elucidation of their functions to the discovery and development of lead compounds with desired properties. Computational tools offer the advantage of delivering new drug candidates more quickly and at a lower cost. Current trend in drug discovery is shifted from discovery to design, which needs understanding the biochemistry of the disease, pathways, identifying disease causative proteins and then designing compounds that are capable of modulating the role of these proteins. This has become common practice in biopharmaceutical industries. Both experimental and computational methods play significant roles in the drug discovery and development and most of the times run complementing each other. The main aim of computer aided drug design (CADD) is to bring the best chemical entities to experimental testing by reducing costs and late stage attrition.[56]

CADD involves:

a. Computer based methods to make more efficient drug discovery and development process.
b. Building up chemical and biological information databases about ligands and targets/proteins to identify and optimize novel drugs.
c. Devising *in silico* filters to calculate drug likeness or pharmacokinetic properties for the chemical compounds prior to screening to enable early detection of the compounds which are more likely to fail in clinical stages and further to enhance detection of promising entities.

The two major disciplines of CADD which can manipulate modern day drug discovery process and which are capable of accelerating drug discovery are bioinformatics and chemoinformatics [57].

In general:

a. Bioinformatic techniques hold a lot of prospective in target identification (generally proteins/ enzymes), target validation, understanding the protein, evolution and phylogeny and protein modeling.
b. Chemoinformatic techniques hold a lot of prospective in storage management and maintenance of information related to chemical compounds and related properties, and importantly in the identification of novel bioactive compounds. Besides, chemoinformatic methods are extensively

utilized in *in silico* ADMET (Absorption, Distribution, Metabolism, Elimination, Toxicity) prediction and related issues that help in the reduction of the late stage failure of compounds.

Steps involved in *In Silico* Drug Design

1. Target identification

2. Target validation

3. Lead identification

4. Lead optimization

Target identification

Drug Target is a biomolecule which is involved in signaling or metabolic pathways that are specific to a disease process. Biomolecules play critical roles in disease progression by communicating through either protein–protein interactions or protein–nucleic acid interactions leading to the amplification of signaling events and/or alteration of metabolic processes. In structure based drug design, a known 3D structure of the target is the initial step in target identification. This is usually determined either by X-ray crystallography or by NMR to identify its binding site, the so called active site.[58]

Target validation

Validation of such target is necessary to exhibit a sufficient level of confidence and to know their pharmacological relevance to the disease under investigation. This can be performed from very basic levels such as cellular, molecular levels to the whole animal level. To select targets most likely to be useful in the development of new treatments for disease, researchers analyze and compare each drug target to others based on their association with a specific disease and their ability to regulate biological and chemical compounds in the body. Tests are conducted to confirm that interactions with the drug target are associated with a desired change in the behavior of diseased cells. Research scientists can then identify compounds that have an effect on the target selected.[59]

Lead identification

Identification of effective compounds such as inhibitors, modulators or antagonists for such target is called lead identification. A lead compound or substance is one that is believed to have potential to treat disease. Laboratory scientists can compare known substances with new compounds to determine their likelihood of success. Leads are sometimes developed as collections, or libraries, of individual molecules that possess properties needed in a new drug. Testing is then done on each of these molecules to confirm its effect on the drug target.[60]

Lead optimization

Drug design is a refinement process, in this process, successive changes are made on molecular structures in order to improve activity. However, the process needs to get started with some compounds having at least marginal activity. Compounds showing dose-dependent target modulation in terms of a certain degree of confidence are processed further as lead compounds. *In silico* screening of chemical compound databases for the identification of novel chemotypes is termed as Virtual Screening (VS). Virtual screening is employed to reduce the number of compounds to be tested in experimental stages, thereby allowing focusing on more reliable entities for lead discovery and optimization.[61]

1.9.1 Drug Likeness

As defined earlier, "drug-likeness" assesses qualitatively the chance for a molecule to become an oral drug with respect to bioavailability. Drug-likeness was established from structural or physicochemical inspections of development compounds advanced enough to be considered oral drug-candidates.[62]

Lipinski rule of 5 helps in distinguishing between drug like and non-drug like molecules. It predicts high probability of success or failure due to drug likeness for molecules complying with 2 or more of the following rules

1. Molecular mass less than 500 Dalton
2. High lipophilicity (expressed as LogP less than 5)
3. Less than 5 hydrogen bond donors
4. Less than 10 hydrogen bond acceptors
5. Topological Polar Surface Area <140

The violation of 2 or more of these conditions predicts a molecule as a non-orally available drug.

1.9.2 *In-silico* ADMET prediction

Drug development is a fine balance of optimizing drug like properties to maximize efficacy, safety, and pharmacokinetics. Many early stage drug discovery programs focus on identifying molecules that bind to a target of interest. The importance of ADMET properties has led to their consideration in early stage drug development, leading to a significant reduction in the number of compounds that failed in clinical trials due to poor ADMET properties. Ultimately, irrespective of filters, the early ADMET profiling of drug candidates is a crucial component in determining the potential success of a new compound and when integrated into the drug development process can hopefully mitigate the risk of attrition. Experimental evaluation of small-molecule ADMET properties is both time-consuming and expensive and does not always scale between animal models and humans. The evolution of computational approaches to optimize pharmacokinetic and toxicity properties may enable the progression of discovery leads effectively and swiftly to drug candidates. This has led to a need for novel approaches to understand, explore, and predict ADMET properties of small molecules as a way to improve compound quality and success rate.[63]

1.9.3 Molecular docking

Molecular docking may be defined as an optimization problem, which would describe the best fit orientation of a ligand that binds to a particular protein of interest and is used to predict the structure of the intermolecular complex formed between two or more molecules. The ligand is generally a small molecule, which interacts with the target protein‘s binding sites. The protein-ligand interaction is comparable to the lock-and-key principle, in which the lock encodes the protein and the key is grouped with the ligand. The major driving force for binding appears to be hydrophobic interaction through several possible mutual conformations by which binding may occur. Therefore computational approaches aid 'dock' small molecules into the structures of macromolecular targets and 'score' their potential complementarity to binding sites and are widely used in hit identification and lead optimization. In simple terms, using bioinformatics tools, in silico techniques help in identifying potential drug target by: exploring the target structures for possible active sites, generating candidate molecules, docking these molecules with the target, ranking them according to their binding affinities, and further optimizing the molecules to improve binding characteristics. Thus in modern drug designing, molecular docking is routinely used for understanding drug receptor interaction to predict the binding orientation of potential small

molecule drug candidates to their protein targets in order to predict the affinity and activity of the small molecule.[64]

The docking algorithm usually carries out the first part of the docking (predicting binding conformation) and the scoring function associated with the docking program carries out the second part that is binding free energy calculations. In general, there are two key components of molecular docking as follows:

a. Pose prediction: docking algorithms usually perform pose predictions which aim to identify molecular features that are responsible for molecular recognition. Pose predictions are very complex and often difficult to understand when simulated on a computer.

b. Activity prediction: after the pose prediction by docking algorithm, the immediate step in the docking process is activity prediction, which is also termed scoring. Docking score is achieved by the scoring functions associated with the particular docking software. Scoring functions are designed to calculate biological activity by estimating the interactions between the compound and protein target. During the early stages of the docking experiments, scoring was performed based on the simple shape and electrostatic complementarities. However, currently, the docking conformers are often treated with sophisticated scoring methods that include the Van der Waals interactions, electrostatic interactions, solvation effects and entropic effects.[65]

1.10 LIPOSOMES

The name liposome is derived from the two Greek words 'lipos,' meaning fat, and 'soma,' meaning body. Liposomes are microscopic spherical vesicles varying in size from 0.025 micrometers (µm) up to 2.5 µm vesicles and composed of one or more lipid bilayers with an aqueous core. They are formed when the lipids are dispersed in an aqueous medium by stirring, in turn giving rise to population vesicles which may reach a size range. The major structural components of liposomes are phospholipids and cholesterol. The lipid bilayer is composed of phospholipids which have a hydrophilic head group and a hydrophobic tail group. The head is attracted to water, and the tail, which is made of a long hydrocarbon chain, is repelled by water. Phospholipids as the main component of the liposomes can easily get integrated with the skin lipids improving drug penetration and localization in the skin layers. The cholesterol incorporated in the lipid membrane helps in the increasing the stability of liposomes, as well as it reduces the permeability of the membranes. The properties of cholesterol lead to decrease the fluidity or increase the micro viscosity of the bilayer.[66]

In aqueous environments, the hydrophobic tails self-orient, resulting in a spherical structure comprised of an aqueous core surrounded by a lipophilic bilayer membrane. Liposomes are both biocompatible and biodegradable (e.g., at certain pH and temperature), which can be controlled by modifying the lipid composition. Furthermore, liposomes are amenable to various modifications that improve their efficacy as drug delivery carriers. The addition of PEG also increases the hydrophilicity of the liposome and the subsequent stability of the liposomes in aqueous storage. Drugs or other small molecules can be incorporated into the hydrophilic core or encapsulated within the hydrophobic bilayer during fabrication or a combination of both approaches can be used. For delivery to specific cell types/tissues, liposomes can be modified with proteins or small molecules to form targeted bioconjugates. Due to the wide array of drugs and small molecules that can be incorporated within liposomes, liposomes can be used as combinational agents for simultaneous tissue/cellular imaging and drug delivery.[67]

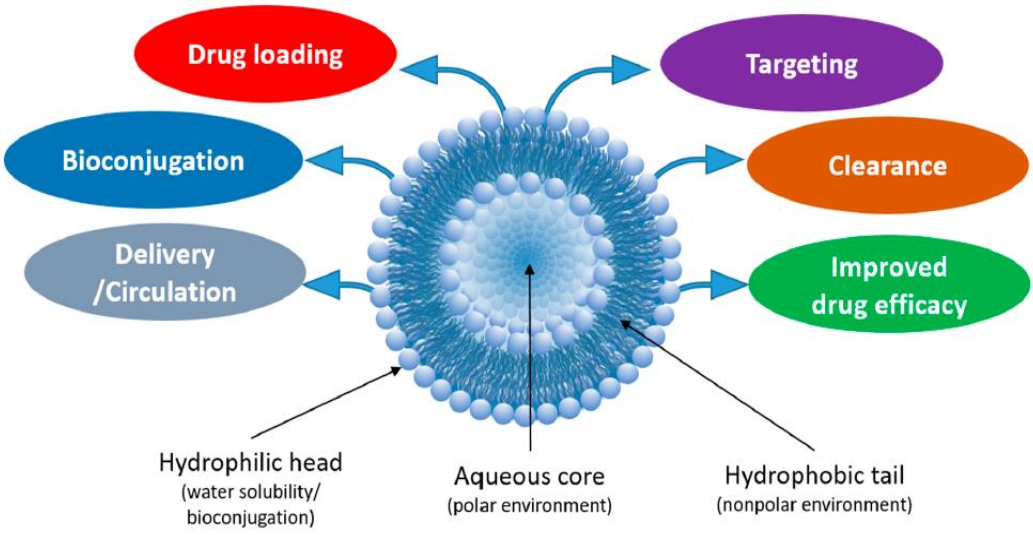


FIGURE 16: Structural features of Liposomes

Generally, based on size the liposomes are classified as: small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and multilamellar vesicles (MLV). The SUV are usually smaller than 50 nm, while LUV are larger than 50 nm. Multilamellar vesicles (MLV) consisted of more bilayers ranged in size from 500 nm to 10000 nm. The unilamellar liposomes have a single phospholipid bilayer sphere enclosing aqueous solution, while the multilamellar vesicles have onion structure which form one inside the other, creating a multilamellar structure of concentric phospholipid spheres separated by layers of water.

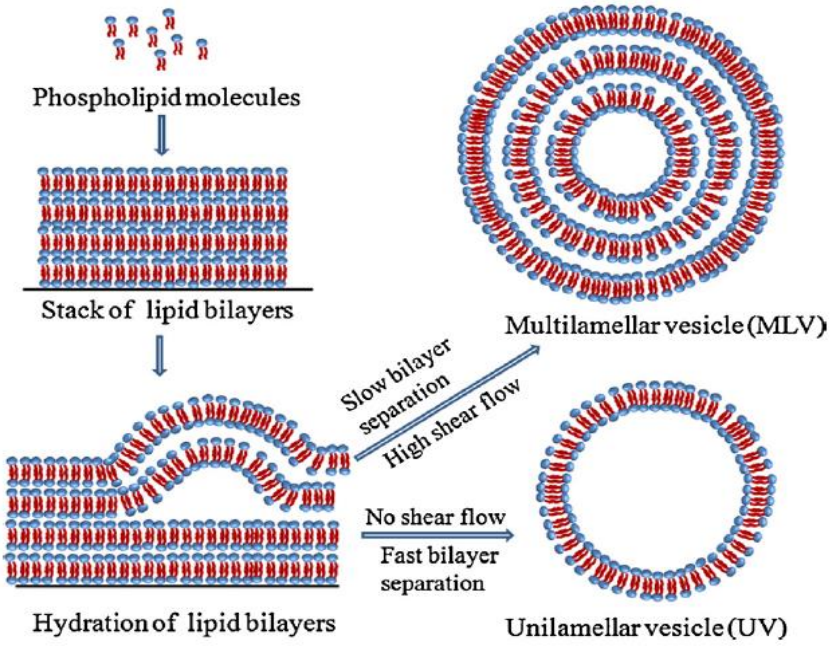


FIGURE 17: Types of Liposomes

1.10.1 Attractive biological properties of liposomes:

- Liposomes are biocompatible.
- Liposomes can entrap water-soluble (hydrophilic) pharmaceutical agents in their internal water compartment and water-insoluble (hydrophobic) pharmaceuticals into the membrane.
- Liposome-incorporated pharmaceuticals are protected from the inactivating effect of external conditions; yet do not cause undesirable side reactions.
- Liposomes provide a unique opportunity to deliver pharmaceuticals into cells or even inside individual cellular compartments.
- Size, charge and surface properties of liposomes can be easily changed simply by adding new ingredients to the lipid mixture before liposome preparation and/or by variation of preparation methods.[68]

1.10.2 Liposomes as successful targeted drug delivery systems in breast cancer

Apart from scale up and their behavior as continuous infusions in body which may pose hurdles in development of liposomes as successful targeted drug delivery systems in breast cancer, they have several advantages listed below:

1. Solubilization: Liposomes solubilize lipophilic drugs that would otherwise be difficult to administer intravenously.

2. Protection: Liposome encapsulated drugs are inaccessible to metabolizing enzymes; conversely body components (erythrocytes or tissues at the site of injection) are not directly exposed to the full dose of the drug.

3. Duration of action: Liposomes prolong drug action by slowly releasing the drug in the body.

4. Directing potential: Targeting options with liposomes change the distribution of the drug through the body thereby enhancing therapeutic response and reducing the dose of the drug associated toxicity.

5. Internalization: Since liposomes undergo endocytosis and phagocytosis they can deliver the drugs intracellularly.[69]

1.11 MDA-MB-231 Cell line profile

The MDA-MB-231 cell line is an epithelial, human breast cancer cell line that was established from a pleural effusion of a 51-year-old caucasian female with a metastatic mammary adenocarcinoma and is one of the most commonly used breast cancer cell lines in medical research laboratories. MDA-MB-231 is a highly aggressive, invasive and poorly differentiated triple-negative breast cancer (TNBC) cell line as it lacks estrogen receptor (ER) and progesterone receptor (PR) expression, as well as HER2 (human epidermal growth factor receptor 2) amplification. Similar to other invasive cancer cell lines, the invasiveness of the MDA-MB-231 cells is mediated by proteolytic degradation of the extracellular matrix.[70]

As a result of lacking ER and PR expression and HER2 amplification, the cell line was initially classed as a 'basal' breast cancer cell line. However, it is now recognised as belonging to the claudin-low molecular subtype as it exhibits down-regulation of claudin-3 and claudinin-4, low expression of the Ki-67 proliferation marker, enrichment for markers associated with the epithelial-mesenchymal transition and the expression of features associated with mammary cancer stem cells (CSCs), such as the CD44+CD24-/low phenotype. In 3D culture, the cell line displays endothelial-like morphology and is distinguished by its invasive phenotype, having stellate projections that often bridge multiple cell colonies.[71]

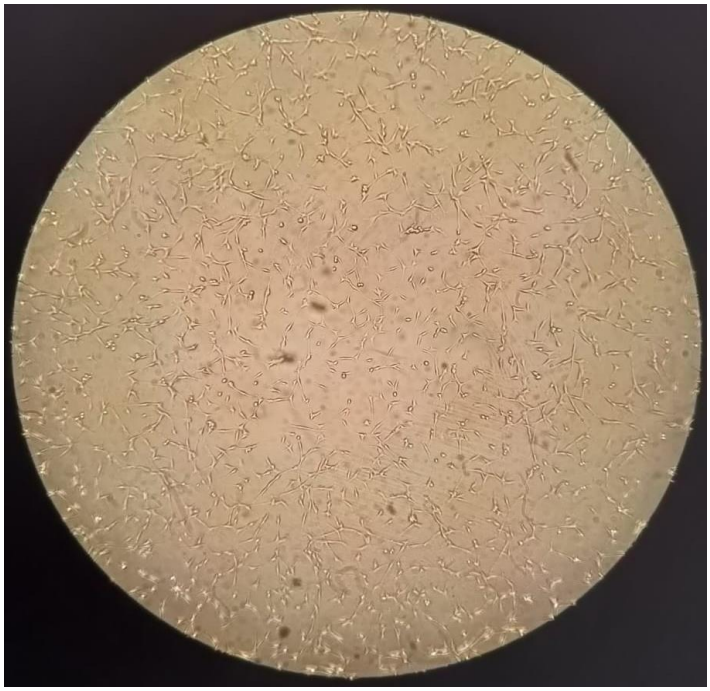


FIGURE 18: Morphology of MDA-MB-231 Cell line

1.11.1 Applications

Triple-negative breast cancer is an aggressive form of breast cancer with limited treatment options. Understanding the molecular basis of triple-negative breast cancer is therefore crucial for effective new drug development and as a result many studies on potentially active agents for this particular type of breast cancer have been conducted using the MDA-MB-231 cell line.

The MDA-MD-231 cell line is well established as a tool for bone metastasis research. Sub-clones of MDA-MB-231 cells that preferentially metastasize either to the bones, brain and lungs of mice following intraventricular injection have also been isolated, thus allowing this cell line to be used in the identification of genes and pathways that are potential mediators of metastasis to specific sites.

2. LITERATURE REVIEW

1. C M O'Higgins et al. (2018) explained about pathophysiology of cancer-related fatigue and current controversies. Fatigue is one of the most common and debilitating cancer symptoms, and is associated with impaired quality of life. The exact pathophysiology of cancer-related fatigue (CRF) is poorly understood, but in any individual, it is likely multifactorial and involves inter-related cytokine, muscular, neurotransmitter, and neuroendocrine changes. Underlying CRF mechanisms proposed include central and peripheral hypotheses. Central mechanisms include hypotheses about cytokine dysregulation, hypothalamic-pituitary-adrenal-axis disruption, circadian rhythm disruption, serotonin, and vagal afferent nerve function while peripheral mechanisms include hypotheses about adenosine triphosphate and muscle contractile properties. The purpose of this article is to provide a narrative review of the literature and present the current controversies in the pathophysiology of CRF, particularly in relation to central and peripheral hypotheses for CRF. An understanding of pathophysiology may facilitate direct and simple therapeutic interventions for those with cancer.[72]

2. Adalberto Miranda-Filho et al. (2020) reviewed about the world cancer patient population (WCPP) and updated standard for international comparisons of population-based survival. This study addresses the need for a global cancer patient-based standard population that adjusts for the expected age structure of different cancers, thus aiding the comparison of survival estimates worldwide. Counts of age-specific incidence for 36 cancer sites in 185 countries for the year 2018 were extracted from IARC's GLOBOCAN database of national estimates. An updated standard entitled the World Cancer Patient Population (WCPP) is presented, derived from the current estimated global numbers of cancer patients that comprises three sets of age-specific weights. Around two-thirds of cancer sites were described by a unique standard, representing the majority of epithelial cancers more often diagnosed at older age groups. The two other standards represent a number of non-epithelial cancer types, and cancers common at younger and older age groups, respectively. The WCPP proposed here provides a contemporary and global means to estimate age-standardised survival for international benchmarking purposes.[73]

3. MA Zaimy et al. (2017) investigated about the new methods in the diagnosis of cancer and gene therapy of cancer based on nanoparticles. Current cancer treatments include surgical

intervention, radiation, and taking chemotherapeutic drugs, which often kill the healthy cells and result in toxicity in patients. Therefore, researchers are looking for ways to be able to eliminate just cancerous cells. Intra-tumor heterogeneity of cancerous cells is the main obstacle on the way of an effective cancer treatment. However, better comprehension of molecular basis of tumor and the advent of new diagnostic technologies can help to improve the treatment of various cancers. Therefore, study of epigenetic changes, gene expression of cancerous cells and employing methods that enable us to correct or minimize these changes is critically important. In this paper, we will review the recent advanced strategies being used in the field of cancer research. [74]

4. Shai libson et al. (2014) reviewed about clinical aspects of breast cancer. Breast cancer is the most frequently diagnosed cancer in women and ranks second among causes for cancer related death in women. The ability to identify and diagnose breast cancer has improved markedly. Treatment decisions which were based in the past predominantly on the anatomic extent of the disease are shifting to the underlying biological mechanisms. Gene array technology has led to the recognition that breast cancer is a heterogeneous disease composed of different biological subtypes, and genetic profiling enables response to chemotherapy to be predicted. Targeted therapy to the oestrogen receptor plays a major role in systemic therapy; pathways responsible for endocrine resistance have been targeted as well. Biological therapy has been developed to target HER2 receptor and combination of antibody drug conjugates linked cytotoxic therapy to HER2 antibodies. Meaningful improvements in survival resulted from the new effective systemic agents and patients with metastasis are likely to have a longer survival. [75]

5. Sergiusz Łukasiewicz et al. (2021) summarized about breast Cancer - epidemiology, Risk Factors, Classification, Prognostic Markers, and Current Treatment Strategies. Breast cancer (BC) is the most frequently diagnosed cancer in women worldwide with more than 2 million new cases in 2020. Its incidence and death rates have increased over the last three decades due to the change in risk factor profiles, better cancer registration, and cancer detection. Invasive BCs comprise wide spectrum tumors that show a variation concerning their clinical presentation, behavior, and morphology. Based on mRNA gene expression levels, BC can be divided into molecular subtypes (Luminal A, Luminal B, HER2-enriched, and basal-like). The eighth edition of TNM classification outlines a new staging system for BC that, in addition to anatomical features, acknowledges

biological factors. Treatment of breast cancer is complex and involves a combination of different modalities including surgery, radiotherapy, chemotherapy, hormonal therapy, or biological therapies delivered in diverse sequences. [76]

6. Olivia Jane Scully et al. (2012) explained about breast cancer metastasis. Breast cancer metastasis accounts for the majority of deaths from breast cancer. Detection of breast cancer metastasis at the earliest stage is important for the management and prediction of breast cancer progression. Emerging techniques using the analysis of circulating tumor cells show promising results in predicting and identifying the early stages of breast cancer metastasis in patients. Additionally, a deeper understanding of the metastatic cascade in breast cancer will be critical for developing therapeutic interventions to combat breast cancer metastasis. In this review, the current and novel methods for detection of breast cancer metastasis, as well as the mechanisms involved in metastasis and the treatment of breast cancer metastasis are discussed. [77]

7. Kimberly S et al. (2017) reviewed about screening methods for breast cancer. This review will give a general overview of the impact of breast cancer, as well as breast cancer risk factors, identification of high-risk groups, screening modalities, and guidelines for screening average-risk, and high-risk individuals, including a case discussion of the primary care provider's approach to Screening. There are several imaging techniques that are available to evaluate the breast. The most widely used and studied modality for breast cancer screening is mammography. Ultrasonography is typically used as an adjunct to mammography for further evaluation of suspicious areas and has been used as a supplement to mammography for women with dense breasts, although data for this practice is incomplete (see discussion below). Magnetic resonance imaging (MRI) is currently used for screening high-risk patients in conjunction with mammography. [78]

8. Khadijeh Barzaman et al. (2020) reviewed about breast cancer- biology, biomarkers, and treatments during the past recent years, various therapies emerged in the era of breast cancer. Breast cancer is a heterogeneous disease in which genetic and environmental factors are involved. Breast cancer stem cells (BCSCs) are the main player in the aggressiveness of different tumors and also, these cells are the main challenge in cancer treatment. Moreover, the major obstacle to achieve an effective treatment is resistance to therapies. There are various types of treatment for

breast cancer (BC) patients. Therefore, in this review, we present the current treatments, novel approaches such as antibody-drug conjugation systems (ADCs), nanoparticles (albumin-, metal-, lipid-, polymer-, micelle-based nanoparticles), and BCSCs-based therapies. Furthermore, prognostic and predictive biomarkers will be discussed also biomarkers that have been applied by some tests such as Oncotype DX, Mamm αPrint, and uPA/PAI-1 are regarded as suitable prognostic and predictive factors in breast cancer. [79]

9. Agnieszka Kolak et al. (2017) explained about primary and secondary prevention of breast cancer. Breast cancer is the most common cancer among women and is the second cancer frequently occurring worldwide of newly-diagnosed cancers. Secondary prevention, comprising diagnostic tests (e.g. mammography, ultrasonography, magnetic resonance imaging, breast self-examination, as well as modern and more precise imaging methods) help the early detection of tumours or lesions predisposing to tumours. Cancer prevention is currently playing a key role in the fight against the disease. Behaviour modification, as well as greater awareness among women regarding breast cancer, may significantly contribute towards reducing the incidence of this cancer. Another important aspect is the number of women undergoing diagnostic tests, which still remains at an unsatisfactory level. [80]

10. Britta Weigelt et al. (2005) summarized about breast cancer metastasis - markers and models breast cancer starts as a local disease, but it can metastasize to the lymph nodes and distant organs. At primary diagnosis, prognostic markers are used to assess whether the transition to systemic disease is likely to have occurred. The prevailing model of metastasis reflects this view it suggests that metastatic capacity is a late, acquired event in tumorigenesis. Others have proposed the idea that breast cancer is intrinsically a systemic disease. New molecular technologies, such as DNA microarrays, support the idea that metastatic capacity might be an inherent feature of breast tumors. These data have important implications for prognosis prediction and our understanding of metastasis. [81]

11. Heather L. McArthur et al. (2007) explained about the breast cancer chemotherapy. Chemotherapy can be an integral component of the adjuvant management strategy for women with early-stage breast cancer. Modern adjuvant strategies now comprises one or more chemotherapy

agents, hormonal maneuvers, immunotherapy agents, or experimental agents. The use of adjuvant chemotherapy is generally based on estimates of an individual's risk of recurrence and the expected benefit of therapy. However, risk-benefit calculations have recently become increasingly sophisticated as a result of advances in genetic testing and molecular marker identification as well as ongoing refinements in chemotherapy strategies. In this article we will review the role of important prognostic and predictive factors and the rationale for adjuvant systemic therapy and modern chemotherapy regimens in the management of women with early stage breast cancer. [82]

12. Savas D. Soysal et al. (2015) investigated about the role of the tumor microenvironment in breast cancer. In recent years, it has been shown that breast cancer consists not only of neoplastic cells, but also of significant alterations in the surrounding stroma or tumor microenvironment. These alterations are now recognized as a critical element for breast cancer development and progression, as well as potential therapeutic targets. Various components of the breast cancer microenvironment, such as suppressive immune cells, soluble factors and altered extracellular matrix, act together to impede effective antitumor immunity and promote breast cancer progression and metastasis. Stromal cells in the breast cancer microenvironment are characterized by molecular alterations and aberrant signaling pathways, some of which are prognostic of clinical outcome. Several new therapies targeting stromal components are in development or undergoing clinical trials. We focus herein on the composition of the breast cancer microenvironment and concomitant molecular alterations, the specific interplay between various cell types and cancer cells, and the clinical implications of these findings. [83]

13. Yiran Liang et al. (2019) summarized about metastatic heterogeneity of breast cancer - molecular mechanism and potential therapeutic targets Breast cancer is one of the most common malignancies among women throughout the world and is the major cause of most cancer-related deaths. Several explanations account for the high rate of mortality of breast cancer, and metastasis to vital organs is identified as the principal cause. However, the underlying molecular mechanism of metastatic heterogeneity of breast cancer remains to be further elucidated. Recently, the advent of novel genomic and pathologic approaches as well as technological breakthroughs in imaging analysis and animal modelling have yielded an unprecedented change in our understanding of the heterogeneity of breast cancer metastasis and provided novel insight for establishing more

effective therapeutics. This review summarizes recent molecular mechanisms and emerging concepts on the metastatic heterogeneity of breast cancer and discusses the potential of identifying specific molecules against tumor cells or tumor microenvironments to the development of metastatic disease and improve the prognosis of breast cancer patients. [84]

14. Karen L et al. (2010) explained about treatment of breast cancer. Understanding breast cancer treatment options can help family physicians care for their patients during and after cancer treatment. This article reviews typical treatments based on stage, histology, and biomarkers. Lobular carcinoma in situ does not require treatment. Ductal carcinoma in situ can progress to invasive cancer and is treated with breast-conserving surgery and radiation therapy without further lymph node exploration or systemic therapy. Stages I and II breast cancers are usually treated with breast-conserving surgery and radiation therapy. Radiation therapy following breast-conserving surgery decreases mortality and recurrence. Choice of adjuvant systemic therapy depends on lymph node involvement, hormone receptor status, ERBB2 (formerly HER2 or HER2/neu) overexpression, and patient age and menopausal status. In general, node-positive breast cancer is treated systemically with chemotherapy, endocrine therapy (for hormone receptor–positive cancer), and trastuzumab (for cancer overexpressing ERBB2). Anthracycline- and taxane-containing chemotherapeutic regimens are active against breast cancer. [85]

15. Xu Cheng et al. (2020) formulated the active-targeting and acid-sensitive pluronic prodrug micelles for efficiently overcoming MDR in breast cancer. Multidrug resistance (MDR) seriously hinders the therapeutic efficacy in clinical cancer treatment. Herein, we reported new polymeric prodrugs micelles with tumor-targeting and acid-sensitivity based on two different pluronic copolymers (F127 and P123) for enhancing tumor MDR reversal and chemotherapy efficiency in breast cancer. Hybrid micelles were composed of phenylboric acid (PBA)-modified F127 (active-targeting group) and doxorubicin (DOX)-grafted P123 (prodrugs group), which accelerated drug release under mildly acidic condition by the cleavage of β-carboxylic amides bonds. In vitro studies demonstrated that FBP-CAD significantly increased cellular uptake and drug concentration in MCF-7/ADR cells through the homing ability of PBA and the anti-MDR effect of P123. In vivo testing further indicated that hybrid micelles facilitated drug accumulation at tumor sites as well as reduced side effects to normal organs. The synergistic effect of active targeting and MDR-

reversing lead to the highest tumor growth inhibition (TGI 78.2%). Thus, these multifunctional micelles provide a feasible approach in nano medicines for resistant-cancer treatment. [86]

16. Ying-Jie Li et al. (2017) reviewed about autophagy and multidrug resistance in cancer. Multidrug resistance (MDR) occurs frequently after long-term chemotherapy, resulting in refractory cancer and tumor recurrence. Therefore, combatting MDR is an important issue. Autophagy, a self-degradative system, universally arises during the treatment of sensitive and MDR cancer. Autophagy can be a double-edged sword for MDR tumors: it participates in the development of MDR and protects cancer cells from chemotherapeutics but can also kill MDR cancer cells in which apoptosis pathways are inactive. Autophagy induced by anticancer drugs could also activate apoptosis signaling pathways in MDR cells, facilitating MDR reversal. Therefore, research on the regulation of autophagy to combat MDR is expanding and is becoming increasingly important. We summarize advanced studies of autophagy in MDR tumors, including the variable role of autophagy in MDR cancer cells. [87]

17. Chenmala Karthika et al. (2020) reviewed about p-glycoprotein efflux transporters and its resistance its inhibitors and therapeutic aspects. P-glycoprotein (P-gp) is an active member of the ATP Binding Cassette (ABC) protein subfamily which effluxes a wide range of therapeutic drugs out of the cells commonly known as multidrug resistance. But its protective action towards the normal cells and efflux of the toxic and foreign substances is remarkable. Hence the efflux of the P-gp is a crucial step to overcome for the success of the therapy and in the drug discovery process. Modification of the action of the P-gp through various inducers, inhibitors or the genetic polymorphism is the commonly used methods. When it comes to the inhibitor part the natural inhibitors use is more safe and economical as compared to the synthetic ones. Here we review at the mechanism of action and the pharmacokinetic profile of P-gp, how the P-gp engaged in the Multidrug resistance, the strategy to overcome from its action by using natural inhibitors and formulation perspectives. [88]

18. Vlasta Nemcova-Furstova et al. (2016) performed characterization of acquired paclitaxel resistance of breast cancer cells and involvement of ABC transporters. The molecular mechanisms underlying the resistance are still unclear. To address this issue, we established paclitaxel-resistant

sublines of the SK-BR-3 and MCF-7 breast cancer cell lines that are capable of long-term proliferation in 100 nM and 300 nM paclitaxel, respectively. Moreover, they demonstrated that it was not possible to establish sublines of SK-BR-3 and MCF-7 cells resistant to this taxaneEmploying mRNA expression profiling of all known human ABC transporters and subsequent Western blot analysis of the expression of selected transporters, they demonstrated that only the ABCB1/PgP and ABCC3/MRP3 proteins were up-regulated in both paclitaxel resistant sublines. They found up-regulation of ABCG2/BCRP and ABCC4 proteins only in paclitaxel-resistant SK-BR-3 cells. In paclitaxel-resistant MCF-7 cells, ABCB4/MDR3 and ABCC2/MRP2 proteins were up-regulated. Silencing of ABCB1 expression using specific siRNA increased significantly, but did not completely restore full sensitivity to both paclitaxel and doxorubicin. Thus they showed a key, but not exclusive, role for ABCB1 in mechanisms of paclitaxel resistance. It suggests the involvement of multiple mechanisms in paclitaxel resistance in tested breast cancer cells. [89]

19. Milica Nedeljković et al. (2021) evaluated the ABCG2, ABCC1 and ABCB1 expression in triple-negative breast cancer. ATP-binding cassette (ABC) transporters are responsible for the efflux of a wide variety of anti-cancer agents and have been implicated in the chemoresistance of various solid tumors. They aimed to investigate the level and frequency of expression of the three most important ABC transporter, ABCG2, ABCC1, and ABCB1, according to breast cancer subtype. They evaluated ABCG2, ABCC1, and ABCB1 protein expressions in 124 primary breast tumors by immunohistochemistry and correlated it to clinicopathological characteristics and outcome. ABCG2 and ABCC1 had a very high level of expression in TNBC that was significantly greater compared to ABCB1 ($p < 0.0001$). ABCB1 expression was associated with TNBC metastatic spread ($p = 0.03$). In contrast, TNBC patients with high ABCG2 expression level had significantly longer disease-free interval ($p = 0.03$) and overall survival ($p = 0.007$). ABCG2, ABCC1, and ABCB1 expression in breast cancer is subtype-specific and associated with triple-negative tumors. The expression of ABCB1 may be useful as a marker of metastatic spread. Moreover, unexpectedly, our results showed a beneficial effect of ABCG2 expression on TNBC clinical behavior. These findings could have implications for the implementation of future TNBC treatment strategies. [90]

20. Rajkumar Chakraborty et al. (2018) done an *in Silico* analysis of nsSNPs in ABCB1 gene affecting breast cancer associated protein P-glycoprotein (P-gp). Breast cancer is one of the most common cancers among women and increased expression of some polymorphic genes. ATP-binding cassette (ABC) genes have been shown to obstruct the treatment of breast cancer by providing resistance to malignant cells from anti-cancer drugs. The present study has incorporated comprehensive bioinformatics analysis to explore the possible disease-associated mutations of ABCB1 gene, a gene that resulted from gene-environment interaction study, and understand their consequential effect on the structural and functional behavior of P-glycoprotein. Two gene variants (R538S and M701R) of P-glycoprotein were selected as potentially detrimental point mutations, and these variants were modeled. Molecular dynamic simulation (MDS) studies unraveled the atomic interactions and motion trajectories of the native as well as the two mutant (R538S and M701R) structures and were predicted to have a deleterious effect on breast cancer associated P-gp. Thus, the present study may broaden the way to design novel potent drugs for overcoming the problems associated with multidrug resistance (MDR) resulting from a change in protein conformation due to a mutation in ABCB1 gene. [91]

21. Sudha Kumari et al. (2010) reviewed about endocytosis unplugged multiple ways to enter the cell. Endocytosis occurs at the cell surface and involves internalization of the plasma membrane (PM) along with its constituent membrane proteins and lipids. Endocytosis is involved in sampling of the extracellular milieu and also serves to regulate various processes initiated at the cell surface. It is also central to the maintenance of PM lipid and protein homeostasis. There are multiple means of internalization that operate concurrently, at the cell surface. With advancement in high-resolution visualization techniques, it is now possible to track multiple endocytic cargo at the same time, revealing a remarkable diversity of endocytic processes in a single cell. A combination of live cell imaging and efficient genetic manipulations has also aided in understanding the functional hierarchy of molecular players in these mechanisms of internalization. Here we provide an account of various endocytic routes, their mechanisms of operation and occurrence across phyla. [92]

22. Kathleen D'Hondt et al. (2021) explained about protein and lipid requirements for endocytosis. Genetic and biochemical studies in yeast and animal cells have led to the

identification of many components required for endocytosis. In this review, we summarize our understanding of the endocytic machinery with an emphasis on the proteins regulating the internalization step of endocytosis and endosome fusion. Even though the overall endocytic machinery appears to be conserved between yeast and animals, clear differences exist. We also discuss the roles of phosphoinositides, sterols, and sphingolipid precursors in endocytosis, because in addition to proteins, these lipids have emerged as important determinants in the spatial and most likely temporal specificity of endocytic membrane trafficking events. [93]

23. Sumadi Lukman Anwar et al. (2015) summarized about caveolin-1 in breast cancer a single molecule regulation of multiple key signaling pathways. Caveolin-1 is a 22-kD trans-membrane protein enriched in particular plasma membrane invaginations known as caveolae. Cav-1 expression is often dysregulated in human breast cancers, being commonly upregulated in cancer cells and downregulated in stromal cells. As an intracellular scaffolding protein, Cav-1, is involved in several vital biological regulations including endocytosis, transcytosis, vesicular transport, and signaling pathways. Several pathways are modulated by Cav-1 including estrogen receptor, EGFR, Her2/neu, TGFβ, and mTOR and represent as major drivers in mammary carcinogenesis. Modification of Cav-1 expression for translational cancer therapy is particularly challenging since numerous signaling pathways might be affected. This review focuses on present understanding of Cav-1 in breast carcinogenesis and its potential role as a new biomarker for predicting therapeutic response and prognosis as well as new target for therapeutic manipulation. [94]

24. Zhong Ming Qian et al. (2002) explained a targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. The membrane transferrin receptor mediated endocytosis or internalization of the complex of transferrin bound iron and the transferrin receptor is the major route of cellular iron uptake. This efficient cellular uptake pathway has been exploited for the site-specific delivery not only of anticancer drugs and proteins, but also of therapeutic genes into proliferating malignant cells that overexpress the transferrin receptors. This is achieved either chemically by conjugation of transferrin with therapeutic drugs, proteins, or genetically by infusion of therapeutic peptides or proteins into the structure of transferrin. The resulting conjugates significantly improve the cytotoxicity and selectivity of the drugs. The coupling of DNA to transferrin via a polycation or liposome serves as a potential alternative to viral vector for

gene therapy. Moreover, the OX26 monoclonal antibody against the rat transferrin receptor offers great promise in the delivery of therapeutic agents across the blood-brain barrier to the brain. [95]

25. Zar Chi Soe et al. (2019) done a research work on transferrin-conjugated polymeric nanoparticle for receptor-mediated delivery of doxorubicin in doxorubicin-resistant breast cancer cells. In this study, a transferrin (Tf)-conjugated polymeric nanoparticle was developed for the targeted delivery of the chemotherapeutic agent doxorubicin (Dox) in order to overcome multi-drug resistance in cancer treatment. The results of our experiments revealed that Dox was successfully loaded inside a transferrin (Tf)-conjugated polymeric nanoparticle composed of poloxamer 407 (F127) and 123 (P123) (Dox/F127&P123-Tf), which produced nano sized particles (~90 nm) with a low Polydispersity index (~0.23). The accelerated and controlled release profiles of Dox from the nanoparticles were characterized in acidic and physiological pH and Dox/F127&P123-Tf enhanced Dox cytotoxicity in OVCAR-3, MDA-MB-231, and MDA-MB-231(R) cell lines through induction of cellular apoptosis. Moreover, Dox/F127&P123-Tf inhibited cell migration and altered the cell cycle patterns of different cancer cells. In vivo study in MDA-MB-231(R) tumor-bearing mice demonstrated enhanced delivery of nanoparticles to the tumor site when coated in a targeting moiety. Therefore, Dox/F127&P123-Tf has been tailored, using the principles of nano therapeutics, to overcome drug-resistant chemotherapy. [96]

26. Soheil Zorofchian Moghadamtousi et al. (2015) reviewed about the *Annona muricata* (annonaceae): a traditional uses, isolated acetogenins and biological activities. *Annona muricata* is a member of the Annonaceae family and is a fruit tree with a long history of traditional use. *A. muricata*, also known as soursop, graviola and guanabana, is an evergreen plant that is mostly distributed in tropical and subtropical regions of the world. A wide array of ethnomedicinal activities is contributed to different parts of *A. muricata*, and indigenous communities in Africa and South America extensively use this plant in their folk medicine. Numerous investigations have substantiated these activities, including anticancer, anticonvulsant, anti-arthritic, antiparasitic, antimalarial, hepatoprotective and antidiabetic activities. Phytochemical studies reveal that Annonaceous acetogenins are the major constituents of *A. muricata*. More than 100 annonaceous acetogenins have been isolated from leaves, barks, seeds, roots and fruits of *A. muricata*. In view

of the immense studies on *A. muricata*, this review strives to unite available information regarding its phytochemistry, traditional uses and biological activities. [97]

27. Islam Rady et al. (2018) reviewed anticancer properties of graviola (Annona muricata). Graviola (Annona muricata) is a small deciduous tropical evergreen fruit tree, belonging to the Annonaceae family, and is widely grown and distributed in tropical and subtropical regions around the world. The reported therapeutic benefits of graviola against various human tumors and disease agents in in vitro culture and preclinical animal model systems are typically tested for their ability to specifically target the disease, while exerting little or no effect on normal cell viability. Over 212 phytochemical ingredients have been reported in graviola extracts prepared from different plant parts. The specific bioactive constituents responsible for the major anticancer, antioxidant, anti-inflammatory, antimicrobial, and other health benefits of graviola include different classes of annonaceous acetogenins (metabolites and products of the polyketide pathway), alkaloids, flavonoids, sterols, and others. It also includes discussion of our current understanding of possible mechanisms of action, with the hope of further stimulating the development of improved and affordable therapies for a variety of ailments. [98]

28. Syed Umar Faruq Syed Najmuddin et al. (2016) done a research work on anti-cancer effect of Annona muricata linn leaves crude extract (amce) on breast cancer cell line. In this study, anti-proliferative and anti-cancer effects of Annona muricata crude extract (AMCE) on breast cancer cell lines were evaluated. A screening of nineteen samples of Annona muricata from different location was determined by MTT assay on breast cancer cell lines (MCF-7, MDA-MB-231, and 4 T1) which revealed a varied potency (IC50) amongst them. Then, based on the IC50 profile from the anti-proliferative assay, further downward assays such as cell cycle analysis, Annexin V/FITC, AO/PI, migration, invasion, and wound healing assay were performed only with the most potent leaf aqueous extract (B1 AMCE) on 4 T1 breast cancer cell line to investigate its anti-cancer effect. At the end of the experiment, histopathology of tumor section, tumor nitric oxide level, tumor malondialdehyde level, clonogenic assay, T cell immunophenotyping, and proteome profiler analysis were performed. Annona muricata crude extract samples exhibited different level of cytotoxicity toward breast cancer cell lines. The selected B1 AMCE reduced the tumor's size and

weight, showed anti-metastatic features, and induced apoptosis in vitro and in vivo of the 4 T1 cells. Furthermore, it decreased the level of nitric oxide and malondialdehyde in tumor while also increased the level of white blood cell, T-cell, and natural killer cell population. The results suggest that, B1 AMCE is a promising candidate for cancer treatment especially in breast cancer and deserves further research as an alternative to conventional drugs while also stressed out the selection of soursop sample which plays a significant role in determining its potential therapeutic effect on cancer. [99]

29. Shashanka K. Prasad et al. (2020) done a research work on phytochemical fractions from Annona muricata seeds and fruit pulp inhibited the growth of breast cancer cells through cell cycle arrest at g0/g1 phase. In this study, we have extracted phytochemicals that exhibited anti-cancer property from the (a) fruit pulp using methanol (AMPM) and water (AMPW); and (b) seeds using methanol (AMSM). Qualitative phytochemical analysis showed the presence of phenolics, tannins, alkaloids, flavonoids, sterols, terpenoids, carbohydrates and proteins in AMPM and AMPW. All three extracts were first checked for in vitro antioxidant and anti-inflammatory properties and then tested for efficacy against MCF-7 and MDA-MB-231. Among these three extracts, AMSM showed the highest antioxidant power as well as ~80% inhibition at 320µg/ml concentration in both cell lines upon treatment for 24h. However, only about 40% inhibition was observed with 320µg/ml AMPM treatment, despite its highest anti-inflammatory potential. Water extract AMPW exhibited about 80% growth inhibition at 50% dilution. Since fruit pulp is the one consumed, the extracts AMPM and AMPW were further tested for apoptosis induction and cell cycle arrest. Analysis of the data showed increased apoptosis and G0/G1 cell cycle arrest upon exposure to AMPM and AMPW. [100]

30. Fei Yuan et al. (2016) done a research work on annosquacin b induces mitochondrial apoptosis in multidrug resistant human breast cancer cell line mcf-7/adr through selectively modulating mapks pathways. Multidrug resistance (MDR) is a major obstacle to efficient therapy of cancers. In recent years, annonaceous acetogenins (ACGs) were reported to have anti-proliferative activity. This study determines the mechanisms of anti-proliferative activity induced by Annosquacin B (AB) against MCF-7/ADR cells. The cytotoxicity of AB at varying concentrations (0.64, 1.6, 4, 10, 25, 62.5, 156.25 lM) on MCF-7/ADR cells was assessed using the MTT assay. Annexin V

FITC/ propidium iodide staining and Acrinidine orange and ethidium bromide (AO/EB) staining were employed to investigate whether AB (14, 7, 3.5 lM) could induce apoptosis in MCF-7/ADR cells. Levels of caspase-3 and caspase-9, Bax, Bcl-2 and MAPKs kinases were evaluated by western blot assay following treatment with various concentrations of AB (3.5, 7, 14 lM) at different time points (0, 0.5, 1, 2, 4, 8, 12 h). MTT assay showed that AB significantly decreased cell viability on MCF-7/ADR (IC50 of 14.69 lM). AB-induced apoptosis in MCF-7/ADR cells through mitochondrial apoptosis pathways. It induced typical apoptosis by morphologic changes; elevate levels of caspase-3, caspase-9 as well as the ratio of Bax/Bcl-2. In addition, AB increased the expression of p-p38 MAPK and decreased the expression of p-JNK, while whether ERK1/2 had an effect on the MCF-7/ADR apoptosis remains to be determined. [101]

31. Heba K Alshaeri et al. (2020) performed a research work on effects of *Annona muricata* extract on triple- negative breast cancer cells mediated through EGFR signaling. To evaluate the antiproliferative activity and the mechanisms of action of *Annona muricata* ethyl acetate (AMEA) extract triple-negative human breast cancer BT-20 cells were used. After the extraction of *Annona muricata*, the extract was exposed to a preparative thin layer chromatography (TLC) plate. From this preparative TLC plate, eight individual bands were collected and removed from the plate and soaked in ethyl acetate. After filtration, all eight fractions were then tested on the BT-20 TNBC cells using the MTS cell viability assay. The expressions of EGFR, p-EGFR, AKT, p-AKT, MAPK, p-MAPK, cyclin D1, and NF-κB p65 were measured using Western blot analysis. The AMEA showed a significant decrease in NF-κB p65 protein expression and BT-20 cell viability, as determined via the MTS assay. Furthermore, the AMEA was subjected to preparative thin layer chromatography (TLC), and eight fractions were obtained. From the eight fractions, only fraction 4 (F4) showed a significant reduction in cell viability in the MTS assay. Immunoblotting analysis revealed that AMEA and F4 formed an antiproliferative effect. These effects were complemented by a downregulation of cyclin D1 assembly, causing cell-cycle arrest at the G1/S phase. Furthermore, NF-κB was measured because of its involvement in the progression of cancers. The antiproliferative influence is produced through EGFR-mediated signaling pathways, which include AKT, MAPK, NF-κB, and cyclin D1 inhibition. Further studies will be required to demonstrate the possible applications of this natural product in breast cancer therapy. [102]

32. Aditi Venkatesh Naik et al. (2020) done a research work on *in vitro* evaluation of *Annona muricata* l. (soursop) leaf methanol extracts on inhibition of tumorigenicity and metastasis of breast cancer cells. The study evaluates the *in-vitro* anti tumorigenic potential of leaf methanol extracts of *A. muricata* (LMAM). The cytotoxic activity was assessed in MCF-7 cells by MTT assay at various concentrations ranging from 25-250µg/mL. MCF-7 cells were treated with 50 and 100µg/mL LMAM for 24h. To detect LMAM-induced apoptosis; Hoescht 33342 staining along with Cell cycle analysis, Annexin-PI probe as well as oxidative stress damage by reactive oxygen species (ROS) measurements were determined using flow cytometric analysis. While, caspase-3 expression levels were studied employing qRT-PCR method. LMAM exhibited significant inhibition of MCF-7 cells with an IC50 value of 85.55µg/mL. Hoescht staining showed marked morphological features characteristic of apoptosis in LMAM treated cells. Cell cycle analysis confirmed proven capability of LMAM showing 30% rise in G1 phase upon treatment with 100µg/mL LMAM, thus inducing cell cycle arrest at G1 phase and a rise in sub G0-G1 population paralleled with a decrease in S phase. Flow cytometric analysis with Annexin V-FITC-PI staining indicated an increase in the early and late apoptotic population with 3.38% and 19.47% rise respectively when treated with 100µg/mL LMAM. Upregulation of caspase-3 was observed with a 2.18 and 32.47 fold increase compared to control in MCF-7 cells cultured at 50µg/mL and 100µg/mL LMAM respectively suggesting caspase-dependent apoptosis. LMAM proved as a potent ethno-chemopreventive agent and a potential lead in cancer treatment attributable to the synergistic interactive properties of phytoconstituents. [103]

33. Nicholas H. Oberlies et al. (1997) done a work on structure-activity relationships of diverse annonaceous acetogenins against multidrug resistant human mammary adenocarcinoma (mcf-7/adr) cells. Fourteen structurally diverse Annonaceous acetogenins were tested for their ability to inhibit the growth of adriamycin resistant human mammary adenocarcinoma (MCF-7/Adr) cells. This cell line is resistant to treatment with adriamycin, vincristine, and vinblastine and is, thus, multidrug resistant (MDR). Among a series of bis-adjacent THF ring acetogenins, those with the stereochemistry of *threo-trans-threo-trans-erythro* (from C-15 to C-24) were the most potent with as much as 250 times the potency of adriamycin. A spacing of 13 carbons between the flanking hydroxyl of the THF ring system and the ç-unsaturated lactone seems to be optimum with a spacing of 11 and 9 carbons being significantly less active. Several single-THF ring compounds were also

quite potent with gigantetrocin A (11) being the most potent compound tested. The acetogenins may, thus, have chemotherapeutic potential, especially with regard to MDR tumors. [104]

34. Ayman Shafei et al. (2017) reviewed the efficacy and toxicity of different doxorubicin nanoparticles for targeted therapy in metastatic breast cancer. Tumor cells develop resistance to chemotherapeutic agents through multiple mechanisms, one of which is over expression of efflux transporters. Various NPs have been investigated to overcome efflux mediated resistance. To date, only liposomal doxorubicin (LD) and pegylated liposomal doxorubicin (PLD) have entered phase II and III clinical trials and FDA- approved for clinical use in MBC. The results derived from: four phase III clinical trials that compared LD with the conventional DOX in naïve MBC patients, and ten non-comparative clinical trials investigated LD and PLD as monotherapy or combination in pretreated MBC. This work confirmed the cardiac tolerability profile of LD and PLD versus DOX, while hematological and skin toxicities were more common. Other DOX-NPs in preclinical trials were discussed in a chronological order. Finally, the modern preclinical development framework for DOX includes exosomal DOX (exo-DOX). Exosomal NPs are non-toxic, non-immunogenic, and can be engineered to have high cargo loading capacity and targeting specificity. These NPs have not been investigated clinically. Our study shows that the full clinical potentiality of DOX-NPs remains to be addressed to move the field forward. [105]

35. Julie Dartier et al. (2017) evaluated the ATP-dependent activity and mitochondrial localization of drug efflux pumps in doxorubicin-resistant breast cancer cells. They hypothesized that, among the mechanisms of drug-resistance acquired by doxorubicin (DOX)-resistant breast cancer cells to maintain cell survival, ATP-dependent drug efflux pumps could be expressed in their mitochondria. The presence and the activity of mitochondrial efflux pumps and their relationship with mitochondrial ATP synthesis were analyzed in DOX-resistant (MCF-7doxR) and -sensitive (MCF-7S) breast cancer cells. Mitochondrial accumulation of DOX (autofluorescence) was decreased when ATP was produced, but only in MCF-7doxR. In these DOX-resistant cells, breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRP1) were expressed and localized in mitochondria (confocal microscopy and confocal spectral imaging studies). In addition, mitochondrial accumulation of DOX was increased by BCRP and MRP1 inhibitors and, to a lower extent, by the mitochondrial ATP synthase inhibitor, oligomycin, in

MCF-7doxR. Both BCRP and MRP1 were localized in mitochondria and participated to the reduction of mitochondrial accumulation of DOX in MCF-7doxR. This process was partly dependent of mitochondrial ATP synthesis. The present study provides novel insights in the involvement of mitochondria in the underlying mechanisms of DOX resistance in breast cancer cells. [106]

36. Hui-na LIU et al. (2018) done a research work on delivery of mitochondriotropic doxorubicin derivatives using self-assembling hyaluronic acid nanocarriers in doxorubicin-resistant breast cancer. They have previously synthesized a doxorubicin (DOX) derivative by conjugating DOX with triphenylphosphonium (TPP) to achieve mitochondrial delivery, which induced higher cytotoxicity in drug-resistant breast cancer cells DOX). The product was analyzed uisng 1H-NMR, 13C-NMR and mass spectrometry. The HA nanocarriers (HA-ionic-TPP-DOX) were shown to self-assemble into spherical nanoparticles, and sensitive to acidic pH in terms of morphology and drug release. Compared with free DOX, HA-ionic-TPP-DOX produced much greater intracellular DOX accumulation and mitochondrial localization, leading to increased ROS production, slightly decreased mitochondrial membrane potential, increased cytotoxicity in MCF-7/ADR cells and enhanced tumor targeting *in vivo* favorable biocompatibility and anti-tumor effects in MCF-7/ADR tumor-bearing nude mice in comparison with the effects of TPP-DOX and DOX, suggesting the potential of HA-ionic-TPP-DOX for the targeted delivery and controlled release of TPP-DOX, which can lead to the sensitization of resistant breast tumors. [107]

37. Carrie J. Lovitt et al. (2018) evaluated the doxorubicin resistance in breast cancer cells is mediated by extracellular matrix proteins. The response of breast cancer cell lines (MCF-7 and MDA-MB-231) to doxorubicin was examined in an in vitro three-dimensional (3D) cell culture model. Cells were cultured with Matrigel™ enabling cellular arrangements into a 3D architecture in conjunction with cell-to-extracellular matrix (ECM) contact. Breast cancer cells cultured in a 3D ECM-based model demonstrated altered sensitivity to doxorubicin, when compared to those grown in corresponding two-dimensional (2D) monolayer culture conditions. Investigations into the factors triggering the observed doxorubicin resistance revealed that cell-to-ECM interactions played a pivotal role. This finding correlated with the up-regulation of pro-survival proteins in 3D ECM-containing cell culture conditions following exposure to doxorubicin. Inhibition of integrin

signalling in combination with doxorubicin significantly reduced breast cancer cell viability. Furthermore, breast cancer cells grown in a 3D ECM-based model demonstrated a significantly reduced proliferation rate in comparison to cells cultured in 2D conditions. Collectively, these novel findings reveal resistance mechanisms which may contribute to reduced doxorubicin sensitivity. [108]

38. Jose Prados et al. (2012) reviewed on doxorubicin-loaded nanoparticles: new advances in breast cancer therapy. Doxorubicin, one of the most effective anticancer drugs, however, its clinical use is restricted by dose-dependent toxicity, the emergence of multidrug resistance and its low specificity against cancer cells. Nanotechnology is a promising alternative to overcome these limitations in cancer therapy as it has been shown to reduce the systemic side-effects and increase the therapeutic effectiveness of drugs. Thus, a suitable nanoparticle system may transport active drugs to cancer cells using the pathophysiology of tumours, especially their enhanced permeability and retention effects, and the tumour microenvironment. In addition, active targeting strategies may allow doxorubicin to reach cancer cells using ligands or antibodies against selected tumour targets. Similarly, doxorubicin resistance may be overcome, or at least reduced, using nanoparticles that are not recognized by P-glycoprotein, one of the main mediators of multidrug resistance, thereby resulting in an increased intracellular concentration of drugs. This paper provides an overview of doxorubicin nanoplatform-based delivery systems and the principal advances obtained in breast cancer chemotherapy. [109]

39. Nesstor Pilco-Ferreto et al. (2016) done a research work on influence of doxorubicin on apoptosis and oxidative stress in breast cancer cell lines. Doxorubicin is a powerful antitumoral drug. It is one of the most active agents for treatment of breast cancer. The aim of the present study was to evaluate the influence of Dox in apoptosis and oxidative stress in the breast cancer cell lines MCF-10F, MCF-7 and MDA-MB- 231. These studies showed that Dox decreased anti-apoptotic Bcl-2 protein expression and affected oxidative stress by increasing hydrogen peroxide production and simultaneously decreasing NF-κB gene and protein expression in MCF-7, a tumorigenic triple-positive cell line. Results also indicated that Dox induced apoptosis by upregulating Bax, caspase-8 and caspase-3 and downregulation of Bcl-2 protein expression. On the contrary, ROS damage decreased by increasing SOD2 gene and protein expression and hydrogen peroxide production

with parallel NF-κB protein expression decrease in MDA-MB-231, a tumorigenic triple-negative breast cancer cell line. It can be concluded that Dox activated apoptosis by inducing proteolytic processing of Bcl-2 family, caspases and simultaneously decreased oxidative stress by influencing ROS damage in MCF-7 and MDA-MB-231 cell lines. [110]

40. Wei Hong et al. (2017) designed a multifunctional micelles against doxorubicin-sensitive and doxorubicin-resistant MCF-7 human breast cancer cells. In this study, three individual functions (active targeting, stimuli-triggered release and endo-lysosomal escape) were evaluated in doxorubicin (DOX)-sensitive MCF-7 cells and DOX-resistant MCF-7/ADR cells by constructing four kinds of micelles with active-targeting (AT-M), passive targeting, pH-triggered release (pHT-M) and endo-lysosomal escape (endoE-M) function, respectively. AT-M demonstrated the strongest cytotoxicity against MCF-7 cells and the highest cellular uptake of DOX due to the folate-mediated endocytosis. The enhanced elimination of DOX from the MCF-7/ADR cells also accounted for the remarkable decrease in cytotoxicity against the cells of AT-M. Three micelles were further evaluated with MCF-7 cells and MCF-7/ADR-resistant cells xenografted mice model. In accordance with the in vitro results, AT-M and endoE-M demonstrated the strongest inhibition on the MCF-7 and MCF-7/ADR xenografted tumor, respectively. Active targeting and active targeting in combination with endo-lysosomal escape have been demonstrated to be the primary function for a nanocarrier against doxorubicin-sensitive and doxorubicin-resistant MCF-7 cells, respectively. These results indicate that the rational design of multifunctional nanocarriers for cancer therapy needs to consider the heterogeneous cancer cells and the primary function needs to be integrated to achieve effective payload delivery. [111]

41. Irfani Aura Salsabila et al. (2020) evaluated a synergistic potential of soursop (*Annona muricata* L.) leaves extract with doxorubicin on 4T1 Cells with antisenescence and anti-reactive-oxygen-species properties. This study aims to unveil the anticancer activity of AME as a cotreatment agent with doxorubicin (dox) on 4T1 cells and AME's relation to senescence. AME was obtained by maceration using 96% ethanol. AME was then subjected to qualitative analysis using TLC compared to quercetin (hRf = 75). Cytotoxic evaluation using the MTT assay revealed that AME showed an IC50 value of 63 μg/mL, while its combination (25 μg/mL) with dox (10 nM) decreased the viability of 4T1 cells to 58 % (CI = 0.15). Flowcytometry using propidium

iodide staining confirmed that AME (13 and 25 μg/mL) caused cell cycle arrest in the G1 phase as a single treatment and G2/M arrest in combination with dox. However, by using the dichloro dihydrofluorescein diacetate staining assay, it turned out that AME at concentrations of 13 and 25 μg/mL decreased intracellular reactive oxygen species (ROS) levels both as a single treatment and in combination with dox. *Senescenceassociated β-galactosidase* assay showed that AME decreased dox-induced senescence. AME alone and in combination with dox (cotreatment) showed cytotoxic effect synergistically on 4T1 cells, but this was not caused by an increase in intracellular ROS levels as well as senescence induction. Therefore, AME showed its potential to be a cotreatment agent with antioxidant property on triple-negative breast cancer cells. [112]

42. Le Anh Vu et al. (2015) explained about *in-silico* drug design a prospective for drug lead discovery. The field of in silico drug design is a rapidly growing area in which many successes have occurred in recent years. The explosion of bioinformatics, chemoinformatics, genomics, proteomics, and structural information has provided hundreds of new targets as well as new ligands. Therefore, in silico drug design represents computational methods and resources that are used to facilitate the opportunities for future drug lead discovery. This review reported a brief history of drug design and summarized the most important steps of in silico drug design strategy for the discovery of new molecular entities. The workflow of the entire virtual designing campaign is discussed, from the choice of a target, the evaluation of a structure of that target, ligand search, receptor theory to molecular docking, virtual high-throughput screening, the pivotal questions to consider in choosing a method for drug lead discovery and evaluation of the drug leads. [113]

43. Deepali Gangrade et al. (2016) reviewed about *in-silico* method in drug discovery. Insilico methods are the leading-edge potential tools for assessing ADME properties. These Machine learning methods have ability in allocating diverse structures and complex mechanisms, are appropriate for prediction of biological activity and therapeutic potency. Insilico is simply; Latin-in silicon (i.e. Performed using computer simulation). These newer Insilico approaches has led to easier and broader discovery of new drug , which in turn affect the success and time for carrying out Clinical trials. The In silico techniques like molecular docking, QSAR, Virtual High throughput screening, Pharmacophore, Fragment based screening are explained. Efforts have been directed at broadening of application scopes and improvement of predictive performance of these

methods. Here the progresses and performances as well as challenges of scrutinizing Insilico method by molecular docking of Tea leaves extracted as anti-malarial (Gallocatecin) in correlation with PLANTS® software has been illustrated as a case study. [114]

44. Aparna Shukla et al. (2019) performed 2D and 3D QSAR modelling, molecular docking and *in-vitro* evaluation studies on 18-glycyrrhetinic acid derivatives against triple negative breast cancer cell line. The present work aimed to develop a predictive 2D and 3D quantitative structure activity relationship (QSAR) models against metastatic TNBC cell line. Five novel derivatives of Glycyrrhetinic acid (GA) named GA-1, GA-2, GA-3, GA 4 and GA-5 were semi-synthesised and screened through the QSAR model. Further, *in-vitro* activities of the derivatives were analysed against human TNBC cell line, MDA-MB-231. The result showed GA-1 exhibit improved cytotoxic activity to that of parent compound (GA). Further, Atomic Property Field (APF) based 3D QSAR and scoring recognise C-30 carboxylic group of GA-1 as major influential factor for its anticancer activity. The significance of C-30 carboxylic group in GA derivatives were also confirmed by molecular docking study against cancer target Glyoxalase-I. Finally, the oral bioavailability and toxicity of GA-1 was assessed by computational ADMET studies. [115]

45. Mohana Krishna Gopisetty et al. (2020) performed a work on androstano-arylpyrimidines a novel small molecule inhibitors of MDR1 for sensitizing multidrug-resistant breast cancer cells. Apart from the numerous physiological functions of MDR1, it is widely known for its role in granting multidrug resistance to cancer cells. This ATP-driven transmembrane protein exports a wide range of chemotherapeutic agents from cancer cells, thereby deterring drugs to reach effective intracellular concentrations. Thus, inhibition of MDR1 expression or function would be a viable option to enhance the accumulation of cytotoxic agents in cancer cells which in turn could improve significantly the success rate of chemotherapy. Although, several pharmacological inhibitors have been designed and tested in the past, due to their unsuccessful translation to clinical application, there is still ongoing research to find suitable compounds to manipulate MDR1 function and potentially overturn multidrug resistance. With additional studies, our work can yield a structural platform for a new generation of small molecule MDR1 inhibitors to sensitize drug resistant cancer cells and at the same time it elucidates the exemplary involvement of endoplasmic reticulum stress in the molecular events to defeat multidrug resistance. [116]

46. Syed Subhani et al. (2015) done a work on homology modelling and molecular docking of MDR1 with chemotherapeutic agents in non-small cell lung cancer. The aim of the study was to examine chemotherapeutic drug binding to MDR1 and the interactions therein. They have used Schro¨dinger suite 2014, to perform homology modelling of human MDR1 based on Mouse MDR1, followed by Induced Fit Docking with Paclitaxel, Docetaxel, Gemcitabine, Carboplatin and Cisplatin drugs. Finally, they evaluated drug binding affinities using Prime/MMGBSA and using these scores we compared the affinities of combination therapies against MDR1. Analysis of the docking results showed Paclitaxel > Docetaxel > Gemcitabine > Carboplatin > Cisplatin as the order of binding affinities, with Paclitaxel having the best docking score. The combination drug binding affinity analysis showed Paclitaxel + Gemcitabine to have the residues best docking score and hence, efficacy. Through our investigation we have identified the Gln 195 and Gln 946 to be more frequently involved in drug binding interactions with MDR1. Our results suggest that, Paclitaxel or combination of Paclitaxel + Gemcitabine could serve as a suitable therapy against MDR1 in NSCLC patients. Thus, our study provides new insight into the possible repurposing of chemotherapeutic drugs in targeting elevated MDR1 levels in NSCLC patients, thereby ensuring better overall outcome. [117]

47. Dharmendra K Yadav et al. (2017) done a work on molecular docking, QSAR and ADMET studies of withanolide analogs against breast cancer. Withanolides are steroidal lactones (highly oxygenated C-28 phytochemicals) and have been reported to exhibit immunomodulatory, anticancer and other activities. A quantitative structure activity relationship (QSAR) model was developed by a forward stepwise multiple linear regression method to predict the activity of withanolide analogs against human breast cancer. The most effective QSAR model for anticancer activity against the SK-Br-3 cell showed the best correlation with activity ($r2$=0.93 and rCV2 =0.90). Similarly, cross-validation regression coefficient (rCV2=0.85) of the best QSAR model against the MCF7/BUS cells showed a high correlation ($r2$=0.91). In particular, compounds CID_73621, CID_435144, CID_301751 and CID_3372729 have a marked antiproliferative activity against the MCF7/BUS cells, while 2,3-dihydrowithaferin A-3-beta-*O*-sulfate, withanolide 5, withanolide A, withaferin A have a high activity against the Sk-Br-3 cells compared to standard drugs 5-fluorouracil (5-FU) and camptothecin. Molecular docking was performed to

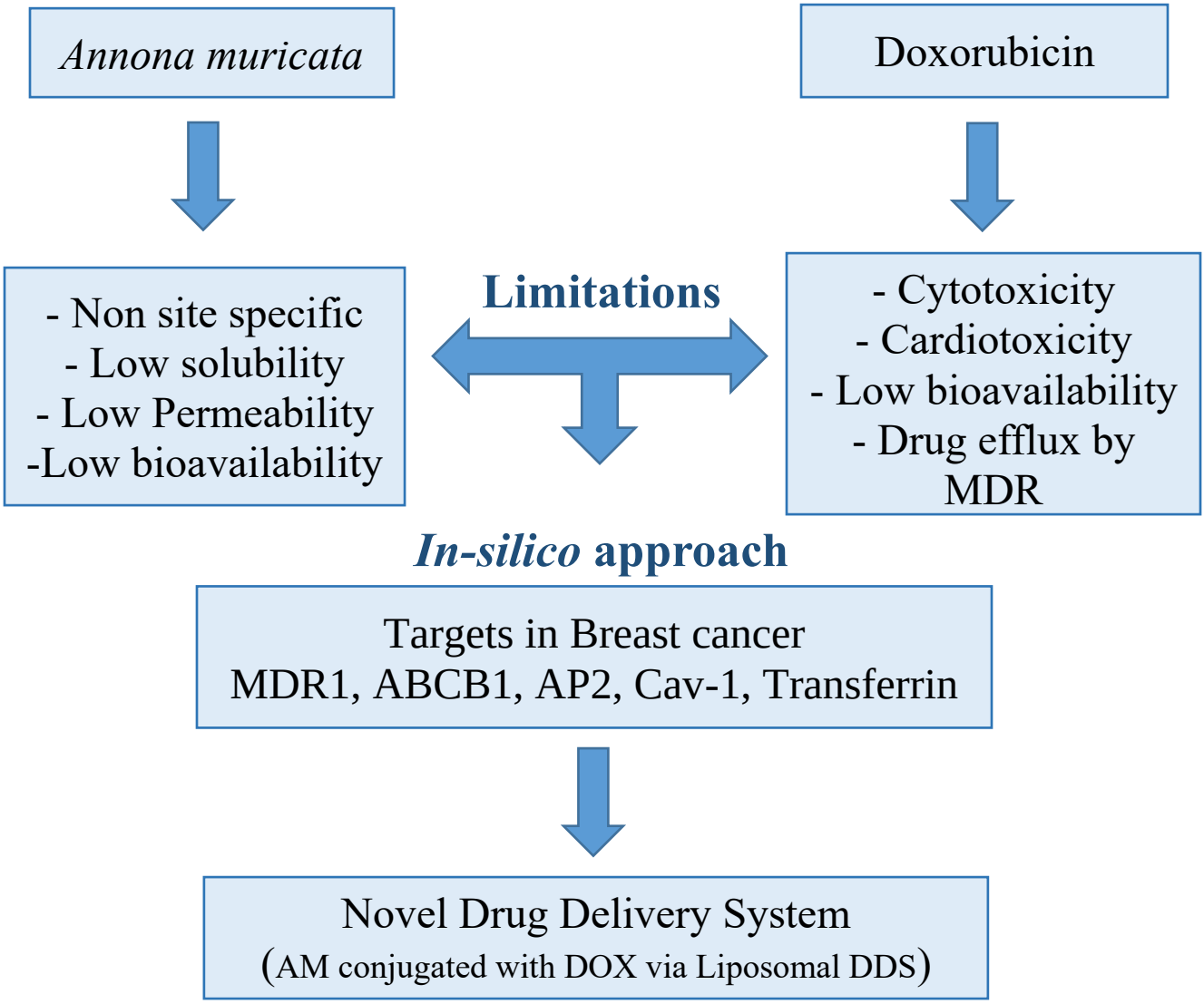
study the binding conformations and different bonding behaviors. The results of the present study may help in the designing of lead compound with improved activity. [118]

48. Hadiza Abdulrahman Lawal et al. (2020) done a work on QSAR, molecular docking, design, and pharmacokinetic analysis of 2-(4-fluorophenyl) imidazol-5-ones as anti-breast cancer drug compounds against MCF-7 cell line. The anti-proliferative activities of Novel series of 2-(4-fluorophenyl) imidazol-5-ones against MCF-7 breast cancer cell line were explored via in-slico studies. Model number one was used in designing new derivative compounds, with higher effectiveness against estrogen positive breast cancer (MCF-7 cell line). The Molecular docking studies between the derivatives and Polo-like kinases (Plk1) receptor proved that the derivatives of 2-(4-fluorophenyl) imidazol-5-ones bind tightly to the receptor, thou ligand 24 and 27 had the highest binding affinities of −8.8 and − 9.1 kcal/mol, which was found to be higher than Doxorubicin with a docking score of −8.0 kcal/mol. These new derivatives of 2-(4 fluorophenyl) imidazol-5-ones shall be excellent inhibitors against (plk1). The pharmacokinetics analysis performed on the new structures revealed that all the structures passed the test and also the Lipinski rule of five, and they could further proceed to pre-clinical tests. They both revealed a revolution in medicine for developing novel anti-breast cancer drugs against MCF-7 cell line. [119]

49. Elizangela Beneval Bento et al. (2016) performed an anti-ulcerogenic activity of the hydroalcoholic extract of leaves of Annona muricata Linnaeus in miceAnnona muricata Linnaeus, popularly known as ‘‘graviola” and also called soursop, is a species typical of countries with a tropical climate, and it is used in folk medicine as an anticancer, analgesic and antispasmodic agent. The aim of the present study was to validate the gastroprotective activity of the hydroalcoholic extract of the leaves of A. muricata (HEAM) and to investigate the underlying mechanisms of action for this effect. Gastric lesions were induced in mice by absolute ethanol, acidified ethanol or indomethacin. Before, the animals were pretreated with saline, omeprazole or HEAM orally at doses of 50–400 mg/kg. To determine the mechanism of action of the extract, we investigated, using specific inhibitors, the involvement of nitric oxide (NO), prostaglan-dins (PGEs), ATP dependent K+ channels and a2-noradrenergic receptors. HEAM showed significant antiulcer activity against lesions induced by absolute ethanol, acidified ethanol or indomethacin, which was mediated by endogenous gastric prostaglandins. [120]

50. Nusrat Chowdhury et al. (2020) done a research work on targeted delivery of doxorubicin liposomes for HER-2+ breast cancer treatment. The goal of this study is to enhance the delivery of doxorubicin by formulating an aptamer-labeled liposomal nanoparticle delivery system that will carry and deliver doxorubicin specifically into Her-2+ breast cancer cells. Twelve liposomal batches were prepared using different saturated (HSPC and DPPC) and unsaturated (POPC and DOPC) lipids by thin film hydration. The liposomes were characterized for their particle size, zeta potential, and drug encapsulation efficiency. The particles were also assessed for in vitro toxicity and DOX delivery into the breast cancer cells. The formulations, F1 through F12, had a small particle size of less than 200 nm and a high entrapment efficiency of about 88 ± 5%. The best formulation, F5, had a particle size of 101 ± 14nm, zeta potential of + 5.63 ± 0.46 mV, and entrapment efficiency of ≈ 93%. The cytotoxicity studies show that the DOX-loaded liposomal formulations are more effective in killing cancer cells than the free DOX in both MCF-7 and SKBR-3 cells. The uptake studies show a significant increase in the uptake of the aptamer-labeled liposomes (i.e., F5) by more than 60% into Her-2+ MCF-7 and SKBR-3 breast cancer cells compare to non-aptamer-labeled nanoparticles. This preliminary study indicates that aptamer-labeled F5 nanoparticles among several batches showed the highest uptake as well as the targeted delivery of doxorubicin into Her-2+ breast cancer cells. Thus, aptamer targeted approach results in substantial reduction in the dose of DOX and improves the therapeutic benefits by promoting the target specificity. [121]

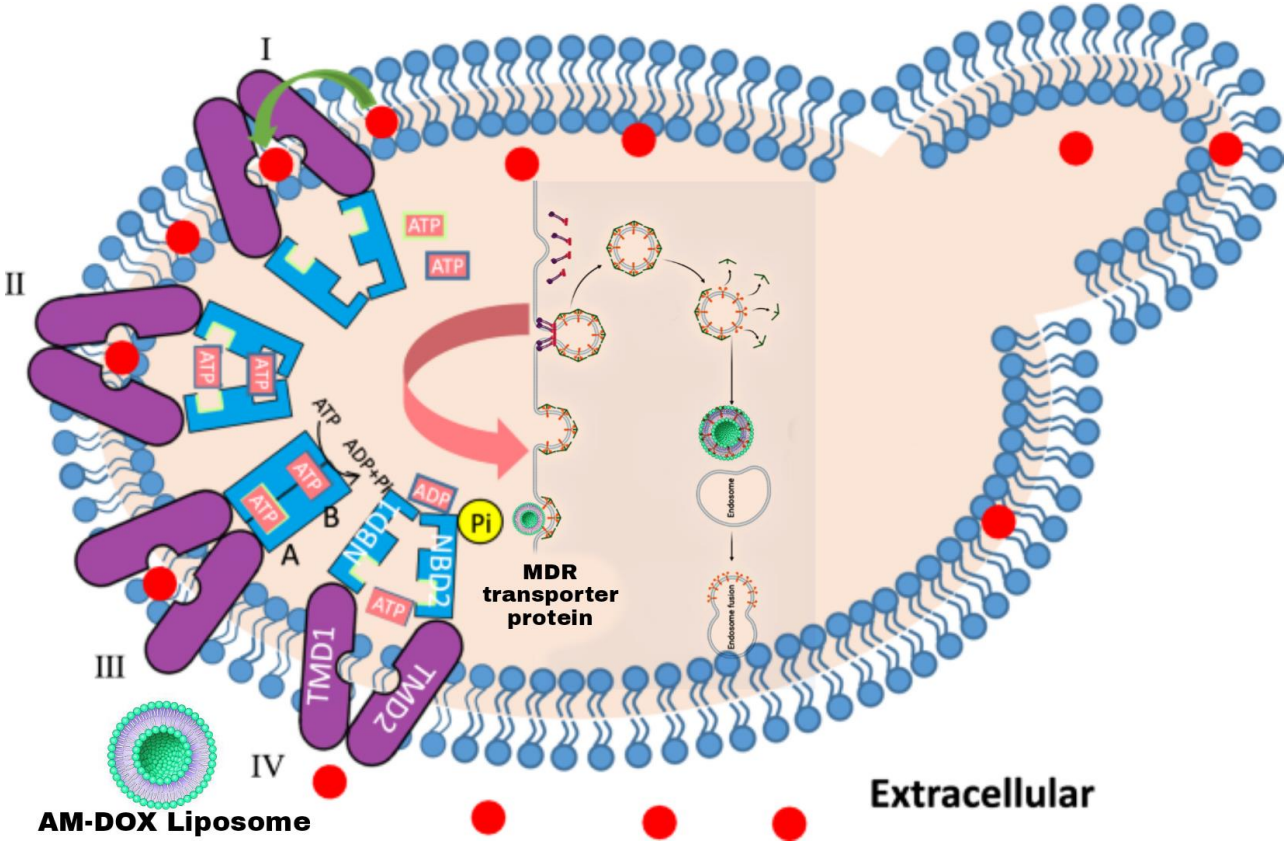
HYPOTHETICAL APPROACH



To overcome above limitations

- Proposed to increase the bioavailability of AM and DOX
- Site specificity may achieved by liposomal conjugates
- Expected to attain Synergistic effect by co-administration of AM&DOX
- Reduction in Cytotoxicity of DOX due to Anti-oxidant property of AM
- Least chance of cardiotoxicity of DOX due to reduction of dosage regimen
- Proposed to inhibition of P-gp efflux by AM which helps to facilitate DOX to deliver into microenvironment of cancer cell via endocytosis, hence we expected to reach maximum therapeutic efficacy and increased drug metabolism of chemotherapeutic agent (Doxorubicin).

GRAPHICAL ABSTRACT



This graphical abstract emphasize that the free drugs are effluxed by P-gp due to the Multi Drug Resistance in cancer cells. To overcome this criteria we conjugated *Annona muricata* leaf extract with Doxorubicin via liposomal drug delivery system. The therapeutic efficacy of drug can be increased by the inhibition of P-gp efflux by phytoconstituents present in *Annona muricata* so that Doxorubicin can deliver into cancer cell through endocytosis mechanism. The identification of combination therapy of plant based extract and chemotherapeutic agent is probably a viable option to treat breast cancer.

3. AIM AND OBJECTIVE

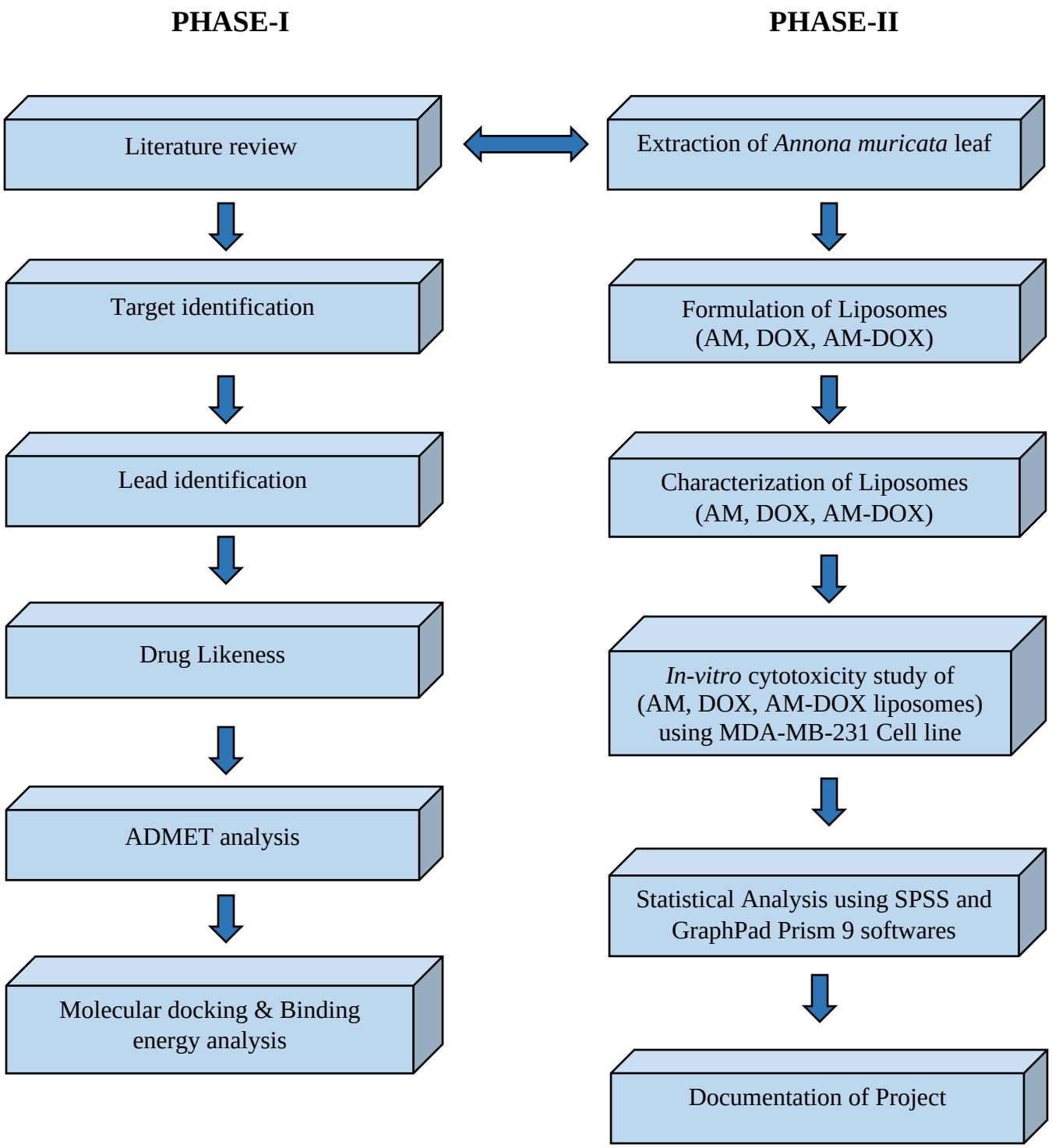
3.1 AIM

The main aim of this study is "To identify and optimize the bioactive lead molecules of Acetogenin derivatives present in *Annona muricata* leaf against the P-gp (P-glycoprotein) efflux proteins (MDR 1, ABCB1) involved in the Breast cancer using *in-silico* molecular docking and to synthesis the liposome conjugated with *Annona muricata* leaf extract and doxorubicin to assess the cytotoxicity using MDA-MB-231 cell line. To achieve this primary aim we fixed some following objectives.

3.2 OBJECTIVES

1. To undergo extensive literature review.
2. To identify the lead molecule from PubChem (https://pubchem.ncbi.nlm.nih.gov/) database as sdf file.
3. To identify the target protein from Protein data bank (https://www.rcsb.org/) as pdb file.
4. To evaluate the Drug likeness for the lead molecules from PubChem.
5. To perform ADMET analysis for the lead molecules by pkCSM tool (http://biosig.unimelb.edu.au/pkcsm/).
6. To perform molecular docking of selected ligands and proteins.
7. To interpret the results of *in-silico* studies.
8. To get authentication certificate of *Annona muricata* from Botanical Survey of India, Tamilnadu agriculture university, Coimbatore.
9. To prepare the hydroalcoholic extract of *Annona muricata* leaf.
10. To synthesis the liposome conjugated with *Annona muricata* leaf extract and Doxorubicin.
11. To perform characterization of liposome.
12. To evaluate the *in-vitro* cytotoxicity using MDA-MB-231 cell line.

4. PLAN OF WORK



5. MATERIALS AND METHODS

PHASE-I

5.1 MATERIALS

The following softwares have been used to perform the *in-silico* evaluations such as Drug likeness, ADMET prediction and Molecular docking.

TABLE 6: List of softwares

S.NO	SOFTWARE NAME	MODE	PURPOSE
1.	PubChem	Online/Free	Collection of Ligands
2.	RCSB database	Online/Free	Collection of Proteins
3.	pkCSM	Online/Free	ADMET prediction, Druglikeness
4.	SPDB viewer	Offline/Free	Protein preparation
5.	BIOVIA Discovery Studio	Offline/Free	a. Ligands collection and clustering b. Study the protein ligand interaction
6.	PyRx	Offline/Free	Docking process
7.	PyMOL	Offline/Free	Building the protein ligand complex

5.2 METHODS

5.2.1 Ligand identification

The three-dimensional structure and two-dimensional structure of the ligands was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) which are listed in the table (7). In order to find its physiochemical properties such as molecular weight, hydrogen bond donors, hydrogen bond acceptors, lipophilicity and topological polar surface area and its properties were compared with Lipinski's rule of five for the evaluation of Drug likeness.

5.2.2 Target identification

The three-dimensional structure of the selected proteins were retrieved from RCSB database (https://www.rcsb.org/) in Protein Data Bank (PDB) format. Drug Target is a biomolecule which is involved in signaling or metabolic pathways that are specific to a disease process PDB id of MDR1, ABCB1, Cav-1, AP2 and Transferrin are 7A65, 6QEX, 4QKV, 2VGL and 1SUV which are listed in the table (8).

TABLE 7: List of Ligands

S.no	Phytoconstituents	Pub Chem CID
1.	Annocatalin	10054251
2.	Annohexocin	10054746
3.	Annomuricin A	157682
4.	Annomuricin B	44575650
5.	Annomuricin C	11758463
6.	Annomuricin-D-One	44559061
7.	Annomuricin E	10371584
8.	Annomutacin	132076
9.	Annonacin A	393471
10.	Annonacin	354398
11.	Annopentocin A	5319155
12.	Annopentocin B	5319163
13.	Annopentocin C	10817542
14.	Cis-Corossolone	11093061
15.	Corossolone	4366126
16.	Doxorubicin	31703
17.	Gigantetronenin	6439484
18.	Iso-Annonacin	128014
19.	Muricatocin B	133072
20.	Muricatocin C	44584147
21.	Muricoreacin	44559047
22.	Murihexocin A	44306793
23.	Murihexocin C	10258454
24.	Murihexocin	44559048

TABLE 8: Targets for molecular docking

S.NO	TARGETS	PDB ID
1.	Multi Drug Resistance protein 1 (MDR1)	7A65
2.	ATP Binding Cassette sub-family B member 1 (ABCB1)	6QEX
3.	Adaptor Protein 2 (AP2)	2VGL
4.	Caveolin-1 (Cav-1) Cavin protein	4QKV
5.	Transferrin	1SUV

5.3 PROCEDURE

5.3.1 DRUG LIKENESS (or) LIPINSKI RULE OF FIVE

The Lipinski's rule of five was published in 1997 by Christopher A. Lipinski and is also known as the Pfizer's rule of five or Rule of five (Ro5). Drug-likeness assesses qualitatively the chance for a molecule to become an oral drug with respect to bioavailability. Drug-likeness was established from structural or physiochemical inspections of development compounds advanced enough to be considered oral drug-candidates. It is a rule of thumb to evaluate the drug-likeness and to determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely orally active drug in humans. Ro5 depends on four simple physiochemical parameter ranges: the molecular weight (MW), which should be less than 500 Dalton, lipophilicity (LogP) less than 5, and number of hydrogen bond donors and acceptors less than 5 and10, respectively. These parameters are connected with intestinal permeability and aqueous solubility and determine the first step of oral bioavailability. These rules explain molecular properties valuable for a drug's pharmacokinetics in the human body, including their absorption, distribution, metabolism, and excretion (ADME). If a ligand fails to fulfill the parameters of Ro5, then it is highly probable that it will cause trouble if ingested. The violation of 2 or more of these conditions predicts a molecule as a non-orally available drug.

5.3.2 ADMET PREDICTION OF LIGANDS

ADMET analysis of ligands (phytoconstituents) are predicted with the help of pkCSM software (http://biosig.unimelb.edu.au/pkcsm/) from this online platform, absorption, distribution, metabolism, excretion, toxicity profile of the drug candidates can be calculated.

ADMET Prediction through pkCSM

1. Open the suitable or convenient web browser and type the URL (http://biosig.unimelb.edu.au/pkcsm/) and enter into the home page.
2. Then click the pkCSM dialogue box.
3. Copy the SMILES string from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and paste in the SMILES box and click the ADMET prediction mode.
4. Results are generated within a minutes and copy the results in word file and save.

Interpretation of ADMET results via pkCSM chart

ABSORPTION

1. Water Solubility

The water solubility of a compound (logS) reflects the solubility of the molecule in water at 25°C. Lipid-soluble drugs are less well absorbed than water-soluble ones, especially when they are enteral. This model is built using experimental water solubility measurements of 1708 molecules.

Result interpretation:

The predicted water solubility of a compound is given as the logarithm of the molar concentration (log mol/L).

2. Caco-2 Permeability

The Caco-2 cell line is composed of human epithelial colorectal adenocarcinoma cells. The Caco-2 monolayer of cells is widely used as an in vitro model of the human intestinal mucosa to predict the absorption of orally administered drugs. This model is based on 674 drug like molecules with Caco-2 permeability values and predicts the logarithm of the apparent permeability coefficient (log Papp; log cm/s).

Result interpretation:

A compound is considered to have a high Caco-2 permeability if it has a Papp > 8 x 10^{-6} cm/s. For the pkCSM predictive model, high Caco-2 permeability would translate in predicted values > 0.90.

3. Intestinal Absorption (Human)

The Intestine is normally the primary site for absorption of a drug from an orally administered solution. This method is built to predict the proportion of compounds that were absorbed through the human small intestine.

Result interpretation:

For a given compound it predicts the percentage that will be absorbed through the human intestine. A molecule with an absorbance of less than 30% is considered to be poorly absorbed.

4. Skin Permeability

Skin permeability is a significant consideration for many consumer products efficacy, and of interest for the development of transdermal drug delivery. This predictor was built using 211 compounds whose in vitro human skin permeability has been measured.

Result interpretation:

It predicts whether if given compound is likely to be skin permeable, expressed as the skin permeability constant logKp (cm/h). A compound is considered to have a relatively low skin permeability if it has a logKp > -2.5.

5. P-glycoprotein substrate

The P-glycoprotein is an ATP-binding cassette (ABC) transporter. It functions as a biological barrier by extruding toxins and xenobiotics out of cells. P-glycoprotein transport screening is performed using transgenic MDR knockout mice and in vitro cell systems. This model was built using 332 compounds that have been characterized for their ability to be transported by P-g protein.

Result interpretation:

The model predicts whether a given compound is likely to be a substrate of Pg protein or not.

6. P-glycoprotein I and II inhibitors

Modulation of P-glycoprotein mediated transport has significant pharmacokinetic implications for Pgp substrates, which may either be exploited for specific therapeutic advantages or result in contraindications. This predictive models were build using 1273 and 1275 compounds that have been characterized for their ability to inhibit P-glycoprotein I and P-glycoprotein II transport, respectively.

Result interpretation:

The predictor will determine is a given compound is likely to be a P-glycoprotein I/II inhibitor.

DISTRIBUTION

1. VDss (Human)

The steady state volume of distribution (VDss) is the theoretical volume that the total dose of a drug would need to be uniformly distributed to give the same concentration as in blood plasma. The higher the VD is, the more of a drug is distributed in tissue rather than plasma. It can be affected by renal failure and dehydration. This predictive model was built using the calculated

steady state volume of distribution (VDss) in humans from 670 drugs. The predicted logarithm of VDss of a given compound is given as the log L/kg.

Result interpretation:

VDss is considered low if below 0.71 L/kg (log VDss < -0.15) and high if above 2.81 L/kg (log VDss > 0.45).

2. Fraction Unbound (Human)

Most drugs in plasma will exist in equilibrium between either an unbound state or bound to serum proteins. Efficacy of a given drug may be affect by the degree to which it binds proteins within blood, as the more that is bound the less efficiently it can traverse cellular membranes or diffuse. This predictive model was built using the measured free proportion of 552 compounds in human blood (Fu).

Result interpretation:

For a given compound the predicted fraction that would be unbound in plasma will be calculated.

3. BBB permeability

The brain is protected from exogenous compounds by the blood-brain barrier (BBB). The ability of a drug to cross into the brain is an important parameter to consider to help reduce side effects and toxicities or to improve the efficacy of drugs whose pharmacological activity is within the brain. Blood-brain permeability is measured in vivo in animal models as logBB, the logarithmic ratio of brain to plasma drug concentrations. This predictive model was built using 320 compounds whose logBB has been experimentally measured.

Result interpretation:

For a given compound, a logBB > 0.3 considered to readily cross the blood-brain barrier while molecules with logBB < −1 are poorly distributed to the brain.

4. CNS permeability

Measuring blood brain permeability can difficult with confounding factors. The blood-brain permeability-surface area product (logPS) is a more direct measurement. It is obtained from in situ brain perfusions with the compound directly injected into the carotid artery. This lacks the systemic distribution effects which may distort brain penetration. This predictive model was built using 153 compounds whose logPS has been experimentally measured.

Result interpretation:

Compounds with a logPS > -2 are considered to penetrate the Central Nervous System (CNS), while those with logPS < -3 are considered as unable to penetrate the CNS.

METABOLISM

1. CYP2D6/CYP3A4 substrate

The cytochrome P450's are responsible for metabolism of many drugs. However inhibitors of the P450's can dramatically alter the pharmacokinetics of these drugs. It is therefore important to assess whether a given compound is likely to be a cytochrome P450 substrate. The two main isoforms responsible for drug metabolism are 2D6 and 3A4. These models were built using 671 compounds whose metabolism by each cytochrome P450 isoform has been measured.

Result interpretation:

The predictor will assess whether a given molecule is likely to be metabolized by either P450.

2. Cytochrome P450 inhibitors

Cytochrome P450 is an important detoxification enzyme in the body, mainly found in the liver. It oxidizes xenobiotics to facilitate their excretion. Many drugs are deactivated by the cytochrome P450's, and some can be activated by it. Inhibitors of this enzyme, such as grapefruit juice, can affect drug metabolism and are contraindicated. It is therefore important to assess a compounds ability to inhibit the cytochrome P450. Models for different isoforms were built (CYP1A2/CYP2C19/CYP2C9/CYP2D6/CYP3A4) using from over 14000 to 18000 compounds whose ability to inhibit the cytochrome P450 has been determined. A compound is considered to be a cytochrome P450 inhibitor if the concentration required to lead to 50% inhibition is less than 10 uM.

Result interpretation:

The predictors will assess a given molecule to determine whether it is likely going to be a cytochrome P450 inhibitor, for a given isoform.

EXCRETION

1. Total Clearance

Drug clearance is measured by the proportionality constant CLtot, and occurs primarily as a combination of hepatic clearance (metabolism in the liver and biliary clearance) and renal clearance (excretion via the kidneys). It is related to bioavailability, and is important for determining dosing rates to achieve steady-state concentrations. This predictor was built using the total clearance data for 398 compounds.

Result interpretation:

The predicted total clearance log (CLtot) of a given compound is given in log (ml/min/kg).

2. Renal OCT2 substrate

Organic Cation Transporter 2 is a renal uptake transporter that plays an important role in disposition and renal clearance of drugs and endogenous compounds. OCT2 substrates also have the potential for adverse interactions with co-administered OCT2 inhibitors. Assessing a candidates potential to be transported by OCT2 provides useful information regarding not only its clearance but potential contraindications. This model was built using 906 compounds whose transport by OCT2 has been experimentally measured.

Result interpretation:

The predictor will assess whether a given molecule is likely to be an OCT2 substrate.

TOXICITY

1. AMES toxicity

The AMES test is a widely employed method to assess a compounds mutagenic potential using bacteria. A positive test indicates that the compound is mutagenic and therefore may act as a carcinogen. This predictive model was built on the results of over 8000 compounds Ames tests.

Result interpretation:

It predicts whether a given compound is likely to be AMES positive and hence mutagenic.

2. Maximum Tolerated Dose

The maximum recommended tolerated dose (MRTD) provides an estimate of the toxic dose threshold of chemicals in humans. The model is trained using 1222 experimental data points from human clinical trials and predicts the logarithm of the MRTD (log mg/kg/day). This will help guide the maximum recommended starting dose for pharmaceuticals in phase I clinical trials, which are currently based on extrapolations from animal data.

Result interpretation:

For a given compound, a MRTD of less than or equal to 0.477 log (mg/kg/day) is considered low, and high if greater than 0.477 log (mg/kg/day).

3. hERG I and II Inhibitors

Inhibition of the potassium channels encoded by hERG (human ether-a-go-go gene) are the principal causes for the development of acquire long QT syndrome - leading to fatal ventricular arrhythmia. Inhibition of hERG channels has resulted in the withdrawal of many substances from the pharmaceutical market. These predictors were built using hERG I and II inhibition information for 368 and 806 compounds, respectively.

Result interpretation:

The predictor will determine if a given compound is likely to be a hERG I/II inhibitor.

4. Rat LD50

It is important to consider the toxic potency of a potential compound. The lethal dosage values (LD50) are a standard measurement of acute toxicity used to assess the relative toxicity of different molecules. The LD50 is the amount of a compound given all at once that causes the death of 50% of a group of test animals.

Result interpretation:

The model was built on over 10000 compounds tested in rats and predicts the LD50 (in mol/kg).

5. Oral Rat Chronic Toxicity

Exposure to low-moderate doses of chemicals over long periods of time is of significant concern in many treatment strategies. Chronic studies aim to identify the lowest dose of a compound that results in an observed adverse effect (LOAEL), and the highest dose at which no adverse effects are observed (NOAEL). This predictor was built using the LOAEL results from 567mpounds.

Result interpretation:

For a given compound, the predicted log Lowest Observed Adverse Effect (LOAEL) in log (mg/kg bw/day) will be generated. The LOAEL results need to be interpreted relative to the bioactive concentration and treatment lengths required.

6. Hepatotoxicity

Drug-induced liver injury is a major safety concern for drug development and a significant cause of drug attrition. This predictor was built using the liver associated side effects of 531 compounds observed in humans. A compound was classed as hepatotoxic if it had at least one pathological or physiological liver event which is strongly associated with disrupted normal function of the liver.

Result interpretation:

It predicts whether a given compound is likely to be associated with disrupted normal function of the liver.

7. Skin Sensitisation

Skin sensitisation is a potential adverse effect for dermally applied products. The evaluation of whether a compound, that may encountered the skin, can induce allergic contact dermatitis is an important safety concern. This predictor was built using 254 compounds which have been evaluated for their ability to induce skin sensitisation.

Result interpretation:

It predicts whether a given compound is likely to be associated with skin sensitisation.

8. T. Pyriformis toxicity

T. Pyriformis is a protozoa bacteria, with its toxicity often used as a toxic endpoint. This method was build using the concentration of 1571 compounds required to inhibit 50% of growth (IGC50).

Result interpretation:

For a given compound, the pIGC50 (negative logarithm of the concentration required to inhibit 50% growth in log ug/L) is predicted, with a value > -0.5 log ug/L is considered toxic.

9. Minnow toxicity

The lethal concentration values (LC50) represent the concentration of a molecule necessary to cause the death of 50% of the Flathead Minnows. This predictive model was built on LC50 measurements for 554 compounds.

Result interpretation:

For a given compound, a log LC50 will be predicted. LC50 values below 0.5 mM (log LC50 < -0.3) are regarded as high acute toxicity.

5.3.3 DOCKING PROCEDURE

Step 1:

- Spdb viewer **(Target protein preparation)**
- Open - (spdbv Application) Click File - Open pdb file
- Select unwanted amino and residues
- Go to Build option - Remove selected residues
- Go to the file – Save - Current layer
- Save the protein as (.pdb) format

Step: 2

- BIOVIA Discovery Studio Visualizer **(Ligands collection & Clustering)**
- File - New - Molecules window
- File - Insert from - Files
- Select all ligands
- File - Save
- Save ligand cluster as (.sdf) format
- Save as type (MDL Mol/ SD Files)

Step: 3

- PyRx Software **(Docking Process)**
- Edit – Preferences…
- In " Work space section – Browse – Click work space folder
- 3 Folders will be created in work space Folder automatically

- Close the PyRx application and again open it
- Click - File - Import - Chemical Table files - SDF
- Click on NEXT
- Select "Ligand Cluster" in "Work space" folder
- All the ligands cluster will be imported
- Then Right click on any one of the ligand and select "Minimize all"
- Again, Right click on any one of the Ligand and select "Convert all to Auto dock ligand (pdbqt)"
- In "Auto dock" section all the ligands will be converted to (.pdbqt) format.
- **Now macromolecules need to be imported**
- File - Load molecules
- Go to (working space) -Then Select the Processed Protein - Open
- Now the Macro molecules needs to be converted to .pdbqt format
- In "molecule" Section Right click the targeted protein
- Auto dock - Make macromolecule
- In the "Auto dock" section the target will be converted to (.Pdbqt) format
- Select "Vina Wizard" – "Select molecules" section
- Then select all the Ligands and Macromolecules in (.pdbqt) format
- Then click "forward"
- Then click "Maximize" to Cover Proteins by grid box
- Then click "Forward"
- The docking process will be Started
- Click on the "Save as comma-separated values (csv)"
- Save the file name as Trial I results .csv
- Hint: Same the file extension in (.csv) format

Step 4:

- PyMOL Application **(Building the Protein ligand complex)**
- File - Open - Working space (Trial 1 Folder) - Protein target
- Trial 1 - Macromolecules – Protein target.pdbqt (or)

- Simply drag the .pdbqt file to PyMOL Window
- Then click "File" - Export molecule - Multi-File - One single file - Save
- File (or) Save name as (protein-ligand complex .pdb)
- Save as type: PDB (*.pdb) files
- Hint: Save the file with extension in (.pdb) format

Step: 5

- BIOVIA discovery studio visualizer **(To Study Protein-Ligand interactions)**
- File – Open – Protein ligand complex
- Tools – Receptor ligand interaction – Define & Edit binding sites – From receptor cavities
- Tools – Receptor ligand interaction – View interaction – Show 2D diagram
- Chart – Ramachandran plot, Hydrophobicity plot, Contact plot – H-bond plot
- File – Save as – Image file

PHASE-II

5.4 MATERIALS

TABLE 9: List of Chemicals and Reagents

S.no	Name of the Chemicals	Manufacturers
1.	LIPOID S PC-3 (Hydrogenated Soy Phosphatidylcholine) and MPEG-2000-DSPE (1,2 disteroyl-sn-glycero-3-phosphoethanolamine-N-methoxy(polyethylene glycol)-2000)	LIPOID, Germany
2.	Doxorubicin, MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	Sigma-Aldrich, Bengaluru
3.	DMEM (Dulbecco's Modified Eagle Medium), FBS (Fetal Bovine Serum), PBS (Phosphate Buffer Saline)	Himedia Labs Ltd, Chennai
4.	Cholesterol (CHO)	Loba Chemie, Mumbai.
5.	Chloroform ($CHCl_3$), Ammonium sulfate ($(NH_4)_2SO_4$), Sucrose, Sodium chloride (NaCl), DMSO (Dimethyl Sulfoxide)	Institutional laboratory facilities
6.	Annona muricata leaf	Locality garden of Sulur, Coimbatore

All the chemicals and reagents used in this study are analytical graded.

5.4.1 INSTRUMENTS

TABLE 10: List of Instruments

S.no	Name of the instruments	Manufacturers
1.	Rotary evaporator	Superfit Laboratory Instruments, India
2.	Vaccum desiccator	Praxor Instruments and Scientific Co, Chennai
3.	Bath sonicator	Labman Scientific Instruments, Chennai
4.	Biosafety Cabinet	Aeromech Equipments, Bengaluru
5.	CO_2 Incubator	Thermo Fischer Scientific, Bengaluru
6.	Microplate reader	Thermo Fischer Scientific, Bengaluru
7.	96 Well Plate	Tarson, Bengaluru
8.	Centrifuge	Remi Lab World, Chennai
9.	Inverted Binocular Microscope	Motic, Mumbai

5.4.2 CELL LINE

MDA-MB-231 cell line was purchased from National Centre for Cell Science (NCCS) Pune, India.

5.5 METHODS

5.5.1 Plant Material and Extract Preparation

Annona muricata leaves were collected from the locality garden of Sulur, Coimbatore, India. The plant material was identified at the Herbarium of Botanical Survey of India, Coimbatore and authenticated a specimen as BSI/SRC/5/23/2021/Tech/301. *Annona muricata* leaves were washed and shade dried. Then leaves were blended into coarse powder with the help of mixer grinder. The powdered leaves are macerated for 3 days with the hydroalcoholic mixture of water and ethanol in 1:1 ratio. Then the Iodine flasks were kept in the Gyratory shaker for 5-6 hours and kept at rest for 15-18 Hours. Then the mixture was filtered using filter paper and the solvent was evaporated by means of Rotary evaporator to obtain the hydroalcoholic extract of *Annona muricata* leaves.

5.5.2 Preparation of AM-DOX Liposome

Liposomes were prepared with Phosphatidylcholine, Cholesterol, and MPEG-DSPE. Briefly, 100 mg Phosphatidylcholine (LIPOID S PC-3), 20 mg MPEG-2000-DSPE and 30 mg Cholesterol were added in 10 mL of HPLC-grade chloroform in a 100-mL round bottom flask. The flask was rotated at 100 rpm while dipping in water bath maintaining a temperature of 55°C. Once the thin film was formed, Prepared Doxorubicin 1mg/ml solution in 0.9% NaCl and *Annona muricata* leaf extract was added into the round bottom flask using the ammonium sulfate loading method. Then the flask was placed in a vacuum desiccator overnight to remove any organic solvent residue. The resulting film of the lipid polymer mixture was hydrated in 120 mM ammonium sulfate (pH 5.5). Resulting liposomes were sonicated for 15 minutes at a temperature of 55°C. And stored in a refrigerator at 2-8^{0}C. A schematic representation of the preparation of AM-DOX liposome has been shown in Figure (19).

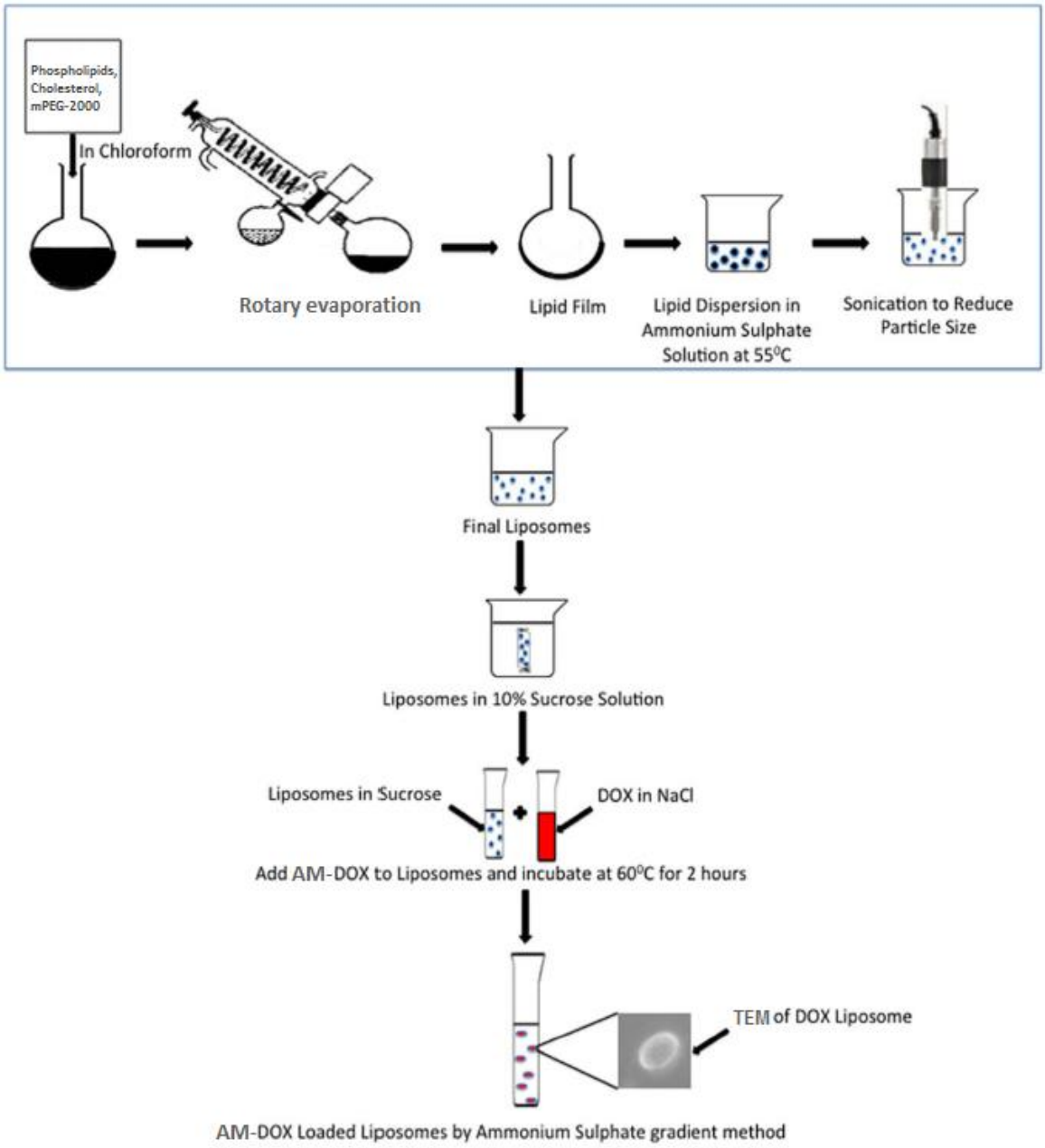


FIGURE 19: Preparation of AM-DOX loaded liposome

5.5.3 Preformulation Studies

1. Physical Incompatibility studies
2. Chemical Incompatibility studies

1. Physical Incompatibility studies:

Physical incompatibility leads to change in color, odor, and viscosity are caused by interaction between two or more substances which shows; visible physical change, non-uniformity, unacceptable, unpalatable product formation, Insolubility, Immiscibility and Liquefaction of solids.

2. Chemical Incompatibility studies:

The Compatibility of a medication with other drugs and implanted materials is an important factor impacting drug safety and efficacy. A FTIR-spectrophotometer equipped with an attenuated total reflectance (ATR) was used to investigate molecular interactions involved in complex formation between drug and excipients. Vacuum-dried samples were scanned over the infrared range (4000 to 500 cm^{-1}) and characteristic peaks were compared.

5.5.4 Formulation Studies

Characterization of Liposomes:

1. Particle Size Distribution, Polydispersity Index, and Zeta Potential Measurement
2. Morphology
3. Entrapment Efficiency
4. Stability Study

1. Particle Size Distribution, Polydispersity Index, and Zeta Potential Measurement:

The particle size distribution and surface charge of liposomes (0.5 mg/mL) in phosphate-buffered saline (PBS, pH 7.4) were measured by dynamic laser- light scattering (DLS) by Zeta sizer (Nano ZS, Malvern Instruments Ltd., UK). Zeta potential of liposomes was determined by electrophoretic mobility determination using Zeta sizer.

2. Morphology:

Liposomes were placed on copper grid films and stained with 2% (w/v) Phosphotungstic acid for morphological observation by transmission electron microscopy (TEM) (JEM-100CX, JEOL, Japan).

3. Entrapment Efficiency:

The entrapment efficiency of formulations was determined by comparing the drug:lipid ratio of the final product to initial concentrations of the lipid. The lipid bilayer of the liposomes were lysed with Triton X (1%) and Phosphate buffered saline (PBS). The mixture was centrifuged for 60 min at 14000 rpm, the supernatant containing drug was collected. The amount of entrapped drug was determined spectrophotometrically at 217 and 496 nm. The calibration curve was linear in the range of 10–10,000 ng/ml. The drug encapsulation efficiency was defined as the percentage of the amount of drug encapsulated in the liposomes to total amount of drug.

The absorbance was measured and the entrapment efficiency was calculated by using following formula:

$$\%\text{Entrapment Efficiency (EE)} = (CT - CS)/CT \times 100$$

EE is the concentration of entrapped drug (ng/mL), CT is the initial concentration of drug used in formulating the liposomes (ng/mL), CS is the concentration of drug in the supernatant (ng/mL), and EE (%) is the percentage of the drug's entrapment.

4. Stability testing:

The stability problem of liposomes were limited to two aspects: the physical stability against aggregation or fusion of the dispersion and the drug retention (or latency), defined as the percentage of drug that is liposome-associated. We will focus on two compounds: *Annona muricata* (AM) and doxorubicin (DOX). AM is a hydrophilic compound that is assumed to have a low tendency to interact with negatively charged bilayer structures; DOX is an amphiphilic drug that interacts with the bilayer, mainly via an electrostatic interaction with the negative charge inducing phospholipids. For highly lipophilic compounds that strongly interact with the bilayer (e.g. cholesterol), leakage from the bilayer does not occur on storage. For these compounds only physical and chemical stability problems have to be addressed.

5.5.5 *In-vitro* Cytotoxicity assay

Cell Culture

MDA-MB-231 cells (epithelial human breast cancer cell line) was purchased from NCCS, Pune and were cultured in Dulbecco's Modified Eagle Medium (DMEM) is supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1% Sodium bicarbonate, 1% Sodium pyruvate and maintained under an atmosphere of 5% CO_2 at 37^{o}C with the humidity of 80%.

MTT assay

The sample was tested for *in vitro* cytotoxicity, using MDA-MB-231 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cultured MDA-MB-231 cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^5 cells/ml cells/well (100 µL) into 96-well tissue culture plate in DMEM medium containing 10 % FBS, 1% L-Glutamine, 1% Sodium bicarbonate and 1% Sodium pyruvate for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the sample in a serum free DMEM medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After the incubation period, MTT (100 µL of 0.5 mg/ml) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium with MTT (100 µL) were aspirated off the wells and washed with PBS (100 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability was calculated.

5.5.6 Statistical Analysis

The two-way analysis of variance (ANOVA) was performed for statistical analysis of the data using IBM SPSS statistical software version 25. The *p*-value less than ($^{*}p<0.05$) were considered as significant. The IC_{50} values are calculated using GraphPad Prism 9 software.

6. RESULTS AND DISCUSSION

6.1 EVALUATION OF DRUG-LIKENESS OR LIPINSKI RULE OF FIVE

TABLE 11: Drug-likeness profile of ligands

S. No	Ligands	Molecular mass (<=500 dalton)	LogP (<=5)	Hydrogen bond donors (<=5)	Hydrogen bond acceptors (<=10)	Topological Polar Surface Area Å^2 (<=140)	Number of Violation
1.	Annocatalin	596.9	8.4	4	7	116	YES(2)
2.	Annohexocin	628.9	6.3	6	9	157	YES(4)
3.	Annomuricin A	612.9	7.4	5	8	137	YES(2)
4.	Annomuricin B	612.9	7.4	5	8	137	YES(2)
5.	Annomuricin C	612.9	7.4	5	8	137	YES(2)
6.	Annomuricin-D-One	612.9	7.1	4	8	134	YES(2)
7.	Annomuricin E	612.9	7.4	5	8	137	YES(2)
8.	Annomutacin	624.9	9.5	4	7	116	YES(2)
9.	Annonacin A	596.9	8.4	4	7	116	YES(2)
10.	Annonacin	596.9	8.4	4	7	116	YES(2)
11.	Annopentocin A	612.9	7.4	5	8	137	YES(2)
12.	Annopentocin B	612.9	7.4	5	8	137	YES(2)
13.	Annopentocin C	612.9	7.4	5	8	137	YES(2)
14.	Cis-Corossolone	578.9	9.4	2	6	93.1	YES(2)
15.	Corossolone	578.9	9.4	2	6	93.1	YES(2)
16.	**Doxorubicin**	543.5	1.3	6	12	206	YES(4)
17.	Gigantetronenin	622.9	9.1	4	7	116	YES(2)
18.	Iso-Annonacin	596.9	8.1	3	7	113	YES(2)
19.	Muricatocin B	612.9	7.4	5	8	137	YES(2)
20.	Muricatocin C	612.9	7.4	5	8	137	YES(2)
21.	Muricoreacin	628.9	6.3	6	9	157	YES(4)
22.	Murihexocin A	628.9	6.3	6	9	157	YES(4)
23.	Murihexocin C	628.9	6.3	6	9	157	YES(4)
24.	Murihexocin	628.9	6.3	6	9	157	YES(4)

Rule of five depends on the five physiochemical parameters. Molecular mass less than 500 Dalton, High lipophilicity (expressed as LogP less than 5), Less than 5 hydrogen bond donors, Less than 10 hydrogen bond acceptors and Topological Polar Surface Area Å^2 less than 140. The violation of two or more of these conditions predicts a molecule as a non-orally available drug. More over

all the ligands shows two violations, but specifically five phytoconstituents shows four violations, they are Annohexocin, Muricoreacin, Murihexocin A, Murihexocin C, Murihexocin and Standard drug Doxorubicin is also shows four violations. Hence the violation of two or more of these rules predicts a molecule as a non-orally available drug.

6.2 ADMET PREDICTION OF LIGANDS

TABLE 12: Absorption profile of ligands

S.no	Ligands	Water solubility (log mol/L)	Caco2 permeability (log Papp in 10^{-6} cm/s)	Intestinal absorption (% Absorbed)	Skin Permeability (log Kp)
1.	Annocatalin	-4.937	0.434	70.669	-2.726
2.	Annohexocin	-4.173	-0.024	58.943	-2.733
3.	Annomuricin A	-4.592	-0.179	64.729	-2.73
4.	Annomuricin B	-4.592	-0.179	64.729	-2.73
5.	Annomuricin C	-4.592	-0.179	64.729	-2.73
6.	Annomuricin-D-One	-4.585	0.369	65.236	-2.725
7.	Annomuricin E	-4.592	-0.179	64.729	-2.73
8.	Annomutacin	-4.774	0.414	72.004	-2.731
9.	Annonacin A	-4.999	0.429	70.518	-2.729
10.	Annonacin	-4.999	0.429	70.518	-2.729
11.	Annopentocin A	-4.623	-0.072	61.877	-2.728
12.	Annopentocin B	-4.623	-0.072	61.877	-2.728
13.	Annopentocin C	-4.623	-0.072	61.877	-2.728
14.	Cis-Corossolone	-4.997	0.529	87.9	-2.724
15.	Corossolone	-4.997	0.529	87.9	-2.724
16.	**Doxorubicin**	-2.915	0.457	62.372	-2.735
17.	Gigantetronenin	-4.806	0.429	74.245	-2.73
18.	Iso-Annonacin	-4.927	0.472	71.025	-2.726
19.	Muricatocin B	-4.594	-0.06	64.713	-2.73
20.	Muricatocin C	-4.594	-0.06	64.713	-2.73
21.	Muricoreacin	-3.1	-0.094	39.504	-2.734
22.	Murihexocin A	-4.177	-0.087	64.197	-2.732
23.	Murihexocin C	-4.177	-0.087	64.197	-2.732
24.	Murihexocin	-4.177	-0.087	64.197	-2.732

TABLE 12: Absorption profile of ligands

S.no	Ligands	P-glycoprotein substrate (Yes/No)	P-glycoprotein I inhibitor (Yes/No)	P-glycoprotein II inhibitor (Yes/No)
1.	Annocatalin	Yes	Yes	Yes
2.	Annohexocin	Yes	Yes	Yes
3.	Annomuricin A	Yes	Yes	Yes
4.	Annomuricin B	Yes	Yes	Yes
5.	Annomuricin C	Yes	Yes	Yes
6.	Annomuricin-D-One	Yes	Yes	Yes
7.	Annomuricin E	Yes	Yes	Yes
8.	Annomutacin	Yes	Yes	Yes
9.	Annonacin A	Yes	Yes	Yes
10.	Annonacin	Yes	Yes	Yes
11.	Annopentocin A	Yes	Yes	Yes
12.	Annopentocin B	Yes	Yes	Yes
13.	Annopentocin C	Yes	Yes	Yes
14.	Cis-Corossolone	Yes	Yes	Yes
15.	Corossolone	Yes	Yes	Yes
16.	**Doxorubicin**	Yes	No	No
17.	Gigantetronenin	Yes	Yes	Yes
18.	Iso-Annonacin	Yes	Yes	Yes
19.	Muricatocin B	Yes	Yes	Yes
20.	Muricatocin C	Yes	Yes	Yes
21.	Muricoreacin	Yes	Yes	No
22.	Murihexocin A	Yes	Yes	Yes
23.	Murihexocin C	Yes	Yes	Yes
24.	Murihexocin	Yes	Yes	Yes

6.2.1 Predicted Absorption Profile of Ligands

1. Water Solubility:

Water Solubility study reveals that the standard drug Doxorubicin shows more solubility than the Phytoconstituents.

2. Caco-2 Permeability:

Caco-2 permeability study reveals that none of the compounds shows Caco-2 permeability because the compounds does not matches the reference value of (log Papp in 10^{-6} cm/s) > 0.90.

3. Intestinal Absorption (Human):

Intestinal Absorption study reveals that all the compounds are absorbed greater than the reference value of 30%. But specifically two Phytoconstituents shows greater absorption (Crossolone and Cis-Crossolone) shows 87.9% of absorption while standard drug Doxorubicin shows 62.3% of intestinal absorption.

4. Skin Permeability:

Skin Permeability results reveals that all the compounds are considered to have a Skin Permeability from the reference value log Kp > -2.5.

5. P-glycoprotein substrate:

Comparatively all the compounds are considered likely to be a Substrate of (P-gp) P-glycoprotein which is an ATP-binding cassette (ABC) transporter.

6. P-glycoprotein I inhibitors:

P-glycoprotein-I study reveals that all the Phytoconstituents inhibits P-glycoprotein-I while the standard drug Doxorubicin do not inhibits P-glycoprotein-I.

7. P-glycoprotein II inhibitors:

P-glycoprotein-II study reveals that all the Phytoconstituents inhibits P-glycoprotein-II except Muricoreacin and also, the standard drug Doxorubicin do not inhibits P-glycoprotein-II.

TABLE 13: Distribution profile of ligands

S.no	Ligands	VDss (human) (log L/kg)	Fraction unbound (human) (Fu)	BBB permeability (log BB)	CNS permeability (log PS)
1.	Annocatalin	-0.845	0.076	-1.512	-3.406
2.	Annohexocin	-1.066	0.191	-1.544	-3.984
3.	Annomuricin A	-0.976	0.163	-1.586	-3.707
4.	Annomuricin B	-0.976	0.163	-1.586	-3.707
5.	Annomuricin C	-0.976	0.163	-1.586	-3.707
6.	Annomuricin-D-One	-0.952	0.108	-1.696	-3.584
7.	Annomuricin E	-0.976	0.163	-1.586	-3.707
8.	Annomutacin	-0.973	0.105	-1.513	-3.401
9.	Annonacin A	-0.859	0.099	-1.428	-3.43
10.	Annonacin	-0.859	0.099	-1.428	-3.43
11.	Annopentocin A	-0.914	0.137	-1.511	-3.698
12.	Annopentocin B	-0.914	0.137	-1.511	-3.698
13.	Annopentocin C	-0.914	0.137	-1.511	-3.698
14.	Cis-Corossolone	-0.816	0.039	-0.542	-2.975
15.	Corossolone	-0.816	0.039	-0.542	-2.975
16.	**Doxorubicin**	1.647	0.215	-1.379	-4.307
17.	Gigantetronenin	-0.984	0.078	-1.469	-3.393
18.	Iso-Annonacin	-0.844	0.049	-1.538	-3.307
19.	Muricatocin B	-0.982	0.14	-1.485	-3.709
20.	Muricatocin C	-0.982	0.14	-1.485	-3.709
21.	Muricoreacin	-0.271	0.237	-2.247	-3.733
22.	Murihexocin A	-0.996	0.187	-1.696	-3.988
23.	Murihexocin C	-0.996	0.187	-1.696	-3.988
24.	Murihexocin	-0.996	0.187	-1.696	-3.988

6.2.2 Predicted Distribution Profile of Ligands

1. VDss-Volume of Distribution (Human):

Volume of distribution study reveals that all the Phytoconstituents has low Volume of distribution in blood plasma while the standard drug Doxorubicin has high Volume of distribution 1.647 (log L/kg) in blood plasma which lies between the reference value of 0.71 to 2.81 (log VDss > 0.45).

2. Fraction Unbound (Human):

Fraction Unbound study reveals that all the compounds are unbounded in plasma but comparatively one Phytoconstituents shows greater unbound value in plasma (Muricoreacin) 0.237 of human blood (Fu) which shows the unbound state to serum proteins.

3. BBB permeability:

BBB permeability study reveals that all the compounds has poor permeability to cross Blood brain barrier (BBB) from the reference value of logBB > 0.3 considered to readily cross the blood-brain barrier while molecules with logBB < −1 are poorly distributed to the brain. But comparatively two Phytoconstituents do not cross BBB (Crossolone and Cis-Crossolone) shows -0.542 from the reference value of -1 to 0.3.

4. CNS permeability:

CNS permeability study reveals that all the compounds do not possess CNS permeability from the reference value of logPS > -2 are considered to penetrate the Central Nervous System (CNS), while those with logPS < -3 are considered as unable to penetrate the CNS. But specifically two Phytoconstituents has poor CNS permeability (Crossolone and Cis-Crossolone) shows -2.975 lies between the reference values.

TABLE 14: Metabolism profile of ligands

S.no	Ligands	CYP2D6 substrate (Yes/No)	CYP3A4 substrate (Yes/No)	CYP1A2 inhibitor (Yes/No)	CYP2C19 inhibitor (Yes/No)
1.	Annocatalin	No	Yes	No	No
2.	Annohexocin	No	Yes	No	No
3.	Annomuricin A	No	Yes	No	No
4.	Annomuricin B	No	Yes	No	No
5.	Annomuricin C	No	Yes	No	No
6.	Annomuricin-D-One	No	Yes	No	No
7.	Annomuricin E	No	Yes	No	No
8.	Annomutacin	No	Yes	No	No
9.	Annonacin A	No	Yes	No	No
10.	Annonacin	No	Yes	No	No
11.	Annopentocin A	No	Yes	No	No
12.	Annopentocin B	No	Yes	No	No
13.	Annopentocin C	No	Yes	No	No
14.	Cis-Corossolone	No	Yes	No	No
15.	Corossolone	No	Yes	No	No
16.	**Doxorubicin**	No	No	No	No
17.	Gigantetronenin	No	Yes	No	No
18.	Iso-Annonacin	No	Yes	No	No
19.	Muricatocin B	No	Yes	No	No
20.	Muricatocin C	No	Yes	No	No
21.	Muricoreacin	No	No	No	No
22.	Murihexocin A	No	Yes	No	No
23.	Murihexocin C	No	Yes	No	No
24.	Murihexocin	No	Yes	No	No

TABLE 14: Metabolism profile of ligands

S.no	Ligands	CYP2C9 inhibitor (Yes/No)	CYP2D6 inhibitor (Yes/No)	CYP3A4 inhibitor (Yes/No)
1.	Annocatalin	No	No	No
2.	Annohexocin	No	No	No
3.	Annomuricin A	No	No	No
4.	Annomuricin B	No	No	No
5.	Annomuricin C	No	No	No
6.	Annomuricin-D-One	No	No	No
7.	Annomuricin E	No	No	No
8.	Annomutacin	No	No	No
9.	Annonacin A	No	No	No
10.	Annonacin	No	No	No
11.	Annopentocin A	No	No	No
12.	Annopentocin B	No	No	No
13.	Annopentocin C	No	No	No
14.	Cis-Corossolone	No	No	No
15.	Corossolone	No	No	No
16.	**Doxorubicin**	No	No	No
17.	Gigantetronenin	No	No	No
18.	Iso-Annonacin	No	No	No
19.	Muricatocin B	No	No	No
20.	Muricatocin C	No	No	No
21.	Muricoreacin	No	No	No
22.	Murihexocin A	No	No	No
23.	Murihexocin C	No	No	No
24.	Murihexocin	No	No	No

6.2.3 Predicted Metabolism Profile of Ligands

1. CYP2D6 substrate:

All the Phytoconstituents and standard drug Doxorubicin are not likely to be metabolized by the Substrate of CYP2D6, which is an isoforms of cytochrome P450 responsible for drug metabolism.

2. CYP3A4 substrate:

Comparatively all the Phytoconstituents are likely to be metabolized by the Substrate of CYP3A4, but specifically one Phytoconstituent, Muricoreacin and Standard drug Doxorubicin is also not likely to be metabolized by the Substrate of CYP3A4.

3. Cytochrome P450 inhibitors:

Comparatively all the Phytoconstituents and Standard drug Doxorubicin are likely to be metabolized by the Cytochrome P450 enzymes from the different isoforms such as (CYP1A2/CYP2C19/CYP2C9/CYP2D6/CYP3A4) so that the compounds do not affect drug metabolism.

TABLE 15: Excretion profile of ligands

S.no	Ligands	Total Clearance (log ml/min/kg)	Renal OCT2 substrate (Yes/No)
1.	Annocatalin	1.802	No
2.	Annohexocin	1.838	No
3.	Annomuricin A	1.846	No
4.	Annomuricin B	1.846	No
5.	Annomuricin C	1.846	No
6.	Annomuricin-D-One	1.7	No
7.	Annomuricin E	1.846	No
8.	Annomutacin	1.844	No
9.	Annonacin A	1.81	No
10.	Annonacin	1.81	No
11.	Annopentocin A	1.827	No
12.	Annopentocin B	1.827	No
13.	Annopentocin C	1.827	No
14.	Cis-Corossolone	1.74	No
15.	Corossolone	1.74	No
16.	**Doxorubicin**	0.987	No
17.	Gigantetronenin	1.838	No
18.	Iso-Annonacin	1.663	No
19.	Muricatocin B	1.825	No
20.	Muricatocin C	1.825	No
21.	Muricoreacin	1.876	No
22.	Murihexocin A	1.864	No
23.	Murihexocin C	1.864	No
24.	Murihexocin	1.864	No

6.2.4 Predicted Excretion Profile of Ligands

1. Total Clearance:

Comparatively all the Phytoconstituents are considered to have a significant total clearance value ranges from 1.7 to 1.8, whereas one Phytoconstituents, Muricoreacin shows the greater clearance value of 1.876 and the Standard drug Doxorubicin shows the lower clearance value of 0.987.

2. Renal OCT2 substrate:

Comparatively all the Phytoconstituents and Standard drug Doxorubicin are not likely to be a Substrate of Organic Cation Transporter 2 (OCT2) that plays an important role in disposition and renal clearance of drugs and endogenous compounds.

TABLE 16: Toxicity profile of ligands

S.no	Ligands	AMES toxicity (Yes/No)	Max. tolerated dose (human) (log mg/ kg/day)	hERG I inhibitor (Yes/No)	hERG II inhibitor (Yes/No)	Oral Rat Acute Toxicity (LD50) (mol/kg)
1.	Annocatalin	No	-0.066	No	No	1.949
2.	Annohexocin	No	0.23	No	No	1.932
3.	Annomuricin A	No	0.042	No	No	1.888
4.	Annomuricin B	No	0.042	No	No	1.888
5.	Annomuricin C	No	0.042	No	No	1.888
6.	Annomuricin-D-One	No	0.195	No	No	1.731
7.	Annomuricin E	No	0.042	No	No	1.888
8.	Annomutacin	No	-0.09	No	No	2.059
9.	Annonacin A	No	-0.086	No	No	1.981
10.	Annonacin	No	-0.086	No	No	1.981
11.	Annopentocin A	No	0.032	No	No	1.901
12.	Annopentocin B	No	0.032	No	No	1.901
13.	Annopentocin C	No	0.032	No	No	1.901
14.	Cis-Corossolone	No	0.103	No	No	1.917
15.	Corossolone	No	0.103	No	No	1.917
16.	**Doxorubicin**	No	0.081	No	Yes	2.408
17.	Gigantetronenin	No	-0.115	No	No	2.047
18.	Iso-Annonacin	No	0.128	No	No	1.835
19.	Muricatocin B	No	0.028	No	No	1.964
20.	Muricatocin C	No	0.028	No	No	1.964
21.	Muricoreacin	No	-0.757	No	Yes	4.449
22.	Murihexocin A	No	0.254	No	No	1.885
23.	Murihexocin C	No	0.254	No	No	1.885
24.	Murihexocin	No	0.254	No	No	1.885

TABLE 16: Toxicity profile of ligands

S.no	Ligands	Oral Rat Chronic Toxicity (LOAEL) (log mg/kg _bw/day)	Hepato toxicity (Yes/No)	Skin Sensitisation (Yes/No)	*T.Pyriformis* toxicity (log ug/L)	Minnow toxicity (log mM)
1.	Annocatalin	1.777	Yes	No	0.289	-2.063
2.	Annohexocin	1.956	No	No	0.285	0.432
3.	Annomuricin A	1.793	No	No	1.793	-0.893
4.	Annomuricin B	1.793	No	No	0.285	-0.893
5.	Annomuricin C	1.793	No	No	0.285	-0.893
6.	Annomuricin-D-One	2.051	No	No	0.286	-1.071
7.	Annomuricin E	1.793	No	No	0.285	-0.893
8.	Annomutacin	1.632	No	No	0.286	-2.469
9.	Annonacin A	1.724	Yes	No	0.288	-2.01
10.	Annonacin	1.724	Yes	No	0.288	-2.01
11.	Annopentocin A	1.8	No	No	0.286	-0.355
12.	Annopentocin B	1.8	No	No	0.286	-0.355
13.	Annopentocin C	1.8	No	No	0.286	-0.355
14.	Cis-Corossolone	1.009	No	No	0.291	-3.349
15.	Corossolone	1.009	No	No	0.291	-3.349
16.	**Doxorubicin**	3.339	Yes	No	0.285	4.412
17.	Gigantetronenin	1.935	Yes	No	0.286	-1.644
18.	Iso-Annonacin	1.953	No	No	0.289	-2.171
19.	Muricatocin B	1.904	No	No	0.285	-1.019
20.	Muricatocin C	1.904	No	No	0.285	-1.019
21.	Muricoreacin	3.266	No	No	0.285	1.884
22.	Murihexocin A	2.032	No	No	0.285	0.832
23.	Murihexocin C	2.032	No	No	0.285	0.832
24.	Murihexocin	2.032	No	No	0.285	0.832

6.2.5 Predicted Toxicity Profile of Ligands

1. AMES toxicity:

Comparatively all the Phytoconstituents and Standard drug Doxorubicin are considered not to be a mutagenic and therefore not act as a carcinogen.

2. Maximum Tolerated Dose:

All the Phytoconstituents and Standard drug Doxorubicin are analysed for the Maximum Recommended Tolerated Dose (MRTD) which provides an estimate of the toxic dose threshold of chemicals in humans.

3. hERG I Inhibitors:

Comparatively all the Phytoconstituents and Standard drug Doxorubicin are not likely to inhibit hERG I (human ether-a-go-go gene) which are the principal causes for the development of acquire long QT syndrome - leading to fatal ventricular arrhythmia.

4. hERG II Inhibitors:

Comparatively all the Phytoconstituents are not likely to inhibit hERG II but specifically one Phyto constituents, Muricoreacin and Standard drug Doxorubicin is also likely to inhibit hERG II which are the principal causes for the development of acquire long QT syndrome - leading to fatal ventricular arrhythmia.

5. Oral Rat Acute Toxicity (LD50):

Comparatively all the Phytoconstituents and Standard drug Doxorubicin are analysed for the toxic potency of a potential compound. The lethal dosage values (LD50) are a standard measurement of acute toxicity used to assess the relative toxicity of different molecules.

6. Oral Rat Chronic Toxicity (LOAEL):

Comparatively all the Phytoconstituents and Standard drug Doxorubicin are analysed for the lowest dose of a compound that results in an observed adverse effect (LOAEL), and the highest dose at which no adverse effects are observed (NOAEL).

7. Hepatotoxicity:

Comparatively all Phytoconstituents are considered not to be a Hepatotoxic but particularly four Phytoconstituents (Annocatalin, Annonacin, Annonacin A, Gigantetronenin) are have a Hepatotoxic in nature. Standard drug Doxorubicin also produce Hepatotoxicity.

8. Skin Sensitisation:

All the Phytoconstituents and Standard drug Doxorubicin are analysed and does not associated with skin sensitisation. Whether it may encounters the skin or may induce allergic contact dermatitis which is an important safety concern.

9. T. Pyriformis toxicity:

T.Pyriformis results reveals that all the Phytoconstituents and Standard drug Doxorubicin are analysed and does not associated with T. Pyriformis toxicity using the reference value of > -0.5 log ug/L which is considered to be toxic.

10. Minnow toxicity:

Comparativelys all the Phytoconstituents are does not associated with Minnow toxicity but specifically five Phytoconstituents are considered to be high acute toxic (Minnow toxic) such as Annohexocin, Muricoreacin, Murihexocin, Murihexocin A, Murihexocin C and Standard drug Doxorubicin is also considered to be high acute toxic using the reference value of (log LC50 < -0.3) which are regarded as high acute toxicity.

6.3 DOCKING INTERACTION ANALYSIS

The docking studies were done using PyRx software. The Phytoconstituents such as Annocatalin, Annohexocin, Annomuricin A, Annomuricin B, Annomuricin C, Annomuricin-D-One, Annomuricin E, Annomutacin, Annonacin A, Annonacin, Annopentocin A, Annopentocin B, Annopentocin C, Cis-Corossolone, Corossolone, Doxorubicin, Gigantetronenin, Iso-Annonacin, Muricatocin B, Muricatocin C, Muricoreacin, Murihexocin A, Murihexocin C, and Murihexocin were docked with the selective targets such as MDR 1, ABCB1, Cav-1, AP2 and Transferrin. The interaction between ligands and proteins binding affinity values were shown in the Table (17).

TABLE 17: List of binding energy

S.no	Ligands	MDR1	ABCB1	AP2	Cav-1	Transferrin
1.	Annocatalin	-6.6	**-10.5**	**-10**	**-7.6**	-8.3
2.	Annohexocin	-6.7	-9.5	-9.8	-6.2	-8.5
3.	Annomuricin A	-6.9	-9.1	-8.5	-5.3	-8
4.	Annomuricin B	-6.3	-8.2	-7.4	-6.9	**-9.8**
5.	Annomuricin C	-7.5	-9.8	-8.2	-5.7	-9.1
6.	Annomuricin-D-One	-6.2	-7.8	-6.9	-5.3	-8.7
7.	Annomuricin E	-6.8	-8	-8.6	-6.8	-8.1
8.	Annomutacin	-5	-4.4	-6.6	-3.8	-6.1
9.	Annonacin A	-7.3	-9.3	-7.1	-5.7	-9.7
10.	Annonacin	-7	-9.8	-8.1	-6.2	-7.1
11.	Annopentocin A	**-9.2**	-7.9	-9.1	-6.4	-8.5
12.	Annopentocin B	-6.9	-9.5	-9.3	-6.4	-7.9
13.	Annopentocin C	-6.4	-8.1	-8.2	-7.1	-8.5
14.	Cis-Corossolone	-6.1	-6	-7	-5.6	-8.4
15.	Corossolone	-6.2	-8	-6.7	-5.8	-6.4
16.	**Doxorubicin**	-7.7	-9.1	-9.4	-7.4	-9
17.	Gigantetronenin	-6.2	-8.3	-8.7	-6.1	-7.8
18.	Iso-Annonacin	-6.7	-6	-6.4	-4.6	-7.8
19.	Muricatocin B	-5.8	-5	-6.5	-4.7	-7.3
20.	Muricatocin C	-8.3	-10.2	-8.9	-5.8	-9.2
21.	Muricoreacin	-5.4	-7.9	-6.3	-4.7	-6.1
22.	Murihexocin A	-6.5	-7.5	-7.7	-5.2	-7.1
23.	Murihexocin C	-7	-10.3	-9	-5.4	-9
24.	Murihexocin	-8.1	-6.5	-7.1	-5.7	-6.8

6.3.1 INTERACTION BETWEEN MDR 1 AND LIGANDS

MDR1 belong to the P-gp (p-glycoprotein) gene subfamily is mostly seen in humans. Where MDR1 (P-gp) is widely seen all over the body and efflux a wide range of drugs over the plasma membrane. The interaction formed between the selected twenty four ligands and MDR 1 has been visualized using BIOVIA Discovery studio visualizer tool. The 2D and 3D structures were visualized using BIOVIA Discovery studio visualizer tool. The ligands were bonded to proteins with five van der Waals interaction such as ALA A:57, ALA A:58, HIS A:61, ILE A:59, GLY A:62 and with three conventional hydrogen bond such as LEU A:56, GLY A:64, ALA A:63 and with one alkyl bond such as ALA A:128 and with two covalent bond such as ILE A:59, HIS A:61 was shown in the Figure (22). It was observed that the compound Annopentocin A CID: 5319155 shows a better interaction with the MDR 1 protein with the binding energy of -9.2 kcal mol−1.

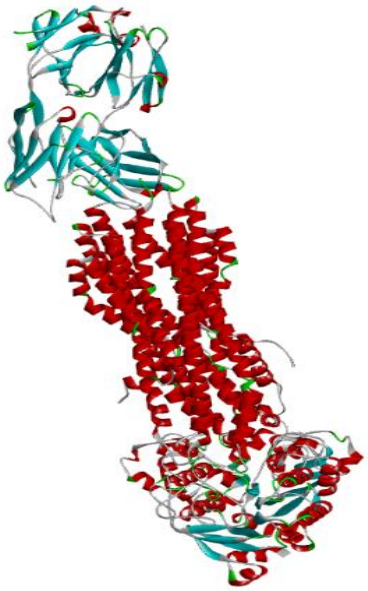


FIGURE 20: Structure of MDR1 Protein

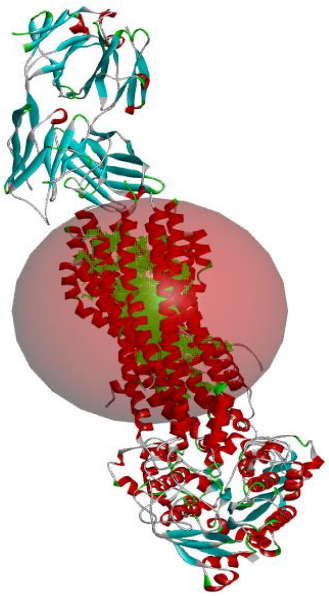


FIGURE 21: Binding site of Ligands with MDR1

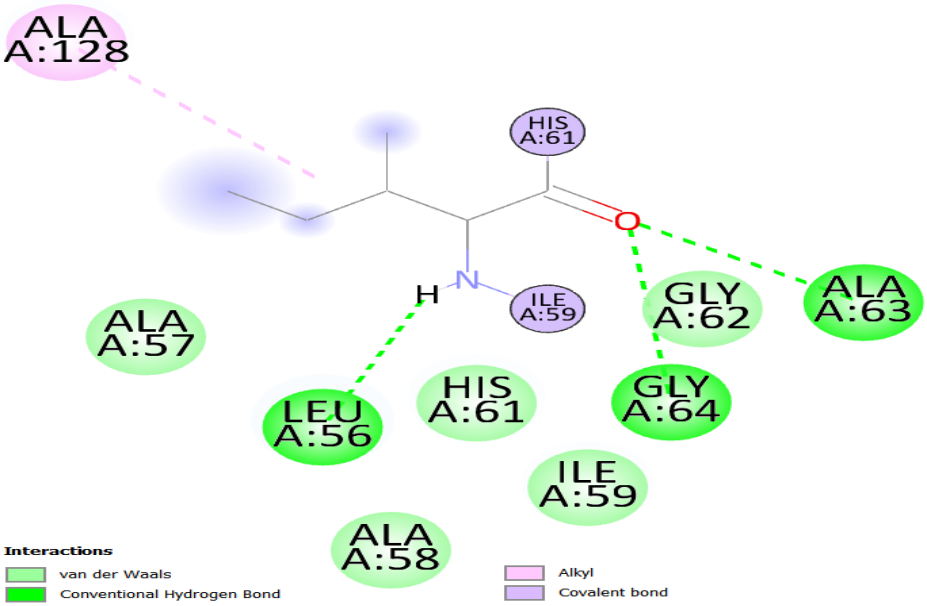


FIGURE 22: 2D complex of MDR1 and Ligands interactions

6.3.2 INTERACTION BETWEEN ABCB1 AND LIGANDS

P-gp is an efflux protein system associated with the ATP binding cassette (ABC) sub-family B membrane. ABC gene indicates the leading family of TM (trans-membrane) protein, originated mainly in the intercellular membrane or the plasma membrane. By using the energy from the ATP the transport mechanism across the cell membrane is initiated. The interaction formed between the selected twenty four ligands and ABCB1 has been visualized using BIOVIA Discovery studio visualizer tool. The 2D and 3D structures were visualized using BIOVIA Discovery studio visualizer tool. The ligands were bonded to proteins with seven van der Waals interaction such as THR A:630, SER A:1269, GLY A:1265, ILE A:1266, TYR A:1267, MET A:1270, VAL A:1271 and with one conventional hydrogen bond such as SER A:1272 and with one pi-alkyl bond such as LYS A:1264 and with two covalent bond such as SER A:1269, TYR A:1267 was shown in the Figure (25). It was observed that the compound Annocatalin CID: 10054251 shows a better interaction with the ABCB1 protein with the binding energy of -10.5 kcal mol−1.

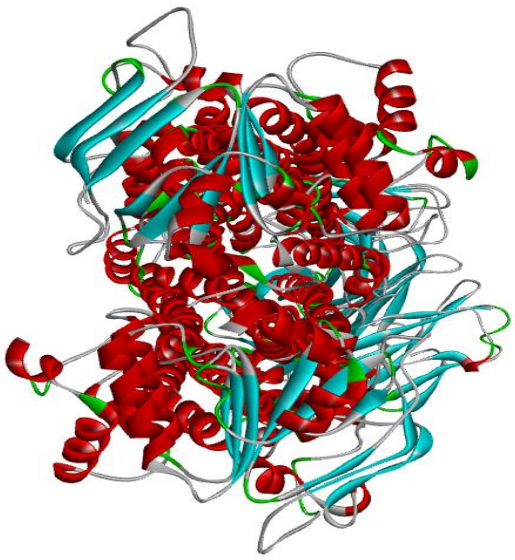


FIGURE 23: Structure of ABCB1 Protein

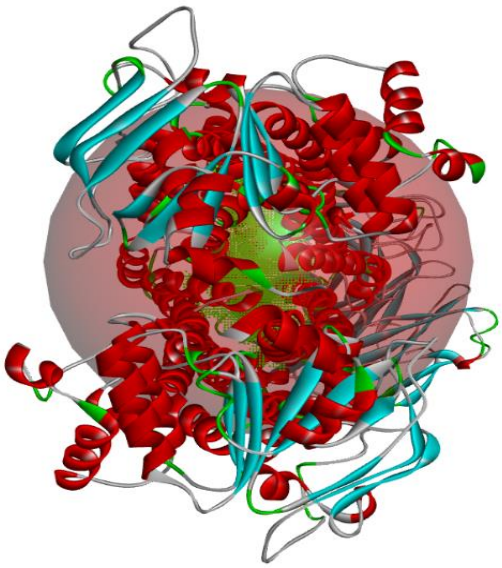


FIGURE 24: Binding site of Ligands with ABCB1

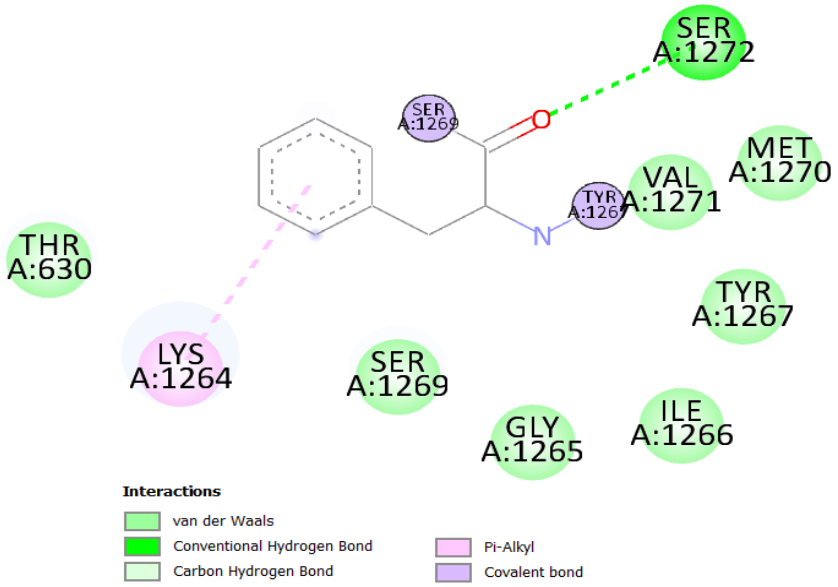


FIGURE 25: 2D complex of ABCB1 and Ligands interactions

6.3.3 INTERACTION BETWEEN AP2 AND LIGANDS

AP2 (Adaptor proteins) functions to promote Clathrin mediated endocytosis. Adaptor proteins contains a variety of protein binding molecules that link protein binding partners together and facilitate the creation of larger signaling complexes. Clathrin coats can spontaneously assemble at the PM (plasma membrane) and are stabilized by interactions with cargo. The interaction formed between the selected twenty four ligands and AP2 has been visualized using BIOVIA Discovery studio visualizer tool. The 2D and 3D structures were visualized using BIOVIA Discovery studio

visualizer tool. The ligands were bonded to proteins with nine van der Waals interaction such as VAL A:428, LEU A:426, LYS A:427, ILE A:424, GLU A:423, GLU A:422, LEU A:413, TYR A:456, VAL A:457 and with two conventional hydrogen bond such as ALA A:429, ARG A:421 and with two alkyl bond such as LEU A:449, ALA A:453 and with two covalent bond such as LEU A:426, ILE A:424 was shown in the Figure (28). It was observed that the compound Annocatalin CID: 10054251 shows a better interaction with the AP2 protein with the binding energy of -10 kcal mol−1.

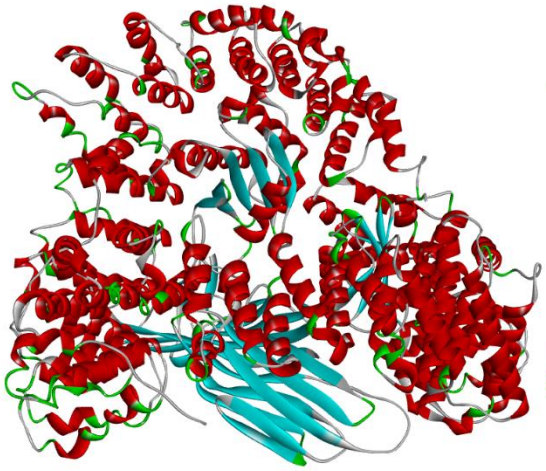


FIGURE 26: Structure of AP2 Protein

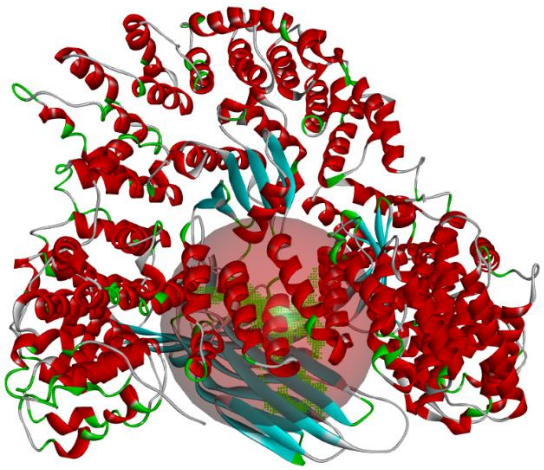


FIGURE 27: Binding site of Ligands with AP2

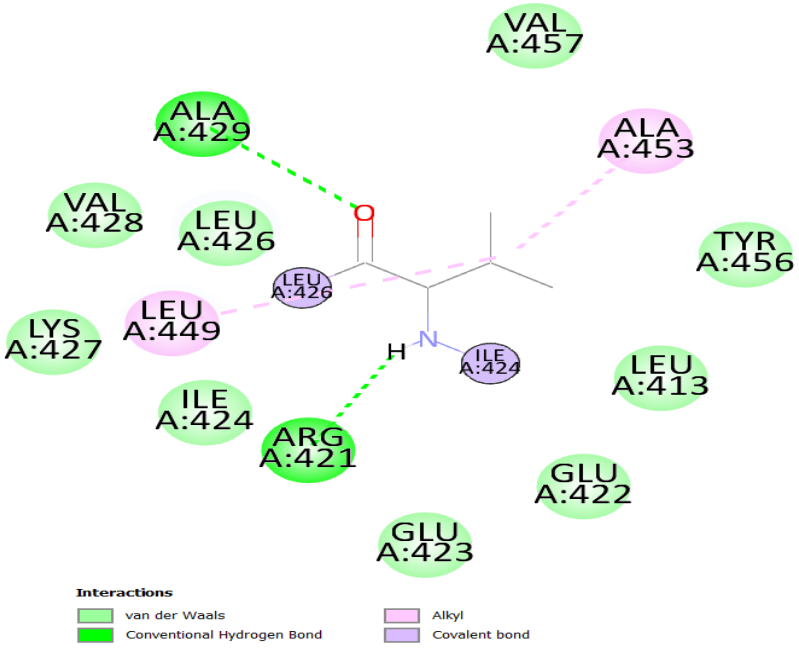


FIGURE 28: 2D complex of AP2 and Ligands interactions

6.3.4 INTERACTION BETWEEN CAV-1 AND LIGANDS

Like Clathrin, another membrane coat at the cell surface is Caveolin. Caveolae were originally described as flask-shaped structures. Caveolae are formed by assembly of caveolins, integral membrane proteins that bind directly to membrane cholesterol. The interaction formed between the selected twenty four ligands and CAV-1 has been visualized using BIOVIA Discovery studio visualizer tool. The 2D and 3D structures were visualized using BIOVIA Discovery studio visualizer tool. The ligands were bonded to proteins with five van der Waals interaction such as GLU A:114, LEU A:113, VAL A:116, ARG A:117, VAL A:116 and with three conventional hydrogen bond such as LEU A:112, LYS A:111, VAL A:119 and with one unfavorable positive-positive bond such as LYS A:118 and with two covalent bond such as VAL A:116, GLU A:114 was shown in the Figure (31). It was observed that the compound Annocatalin CID: 10054251 shows a better interaction with the CAV-1 protein with the binding energy of -7.6 kcal mol−1.

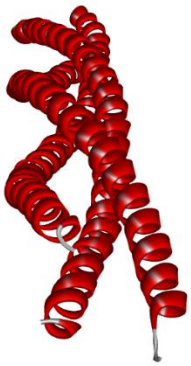


FIGURE 29: Structure of CAV-1 Protein

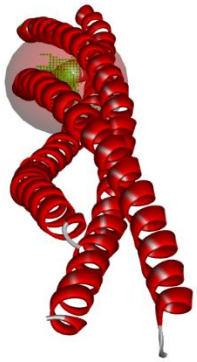


FIGURE 30: Binding site of Ligands with CAV-1

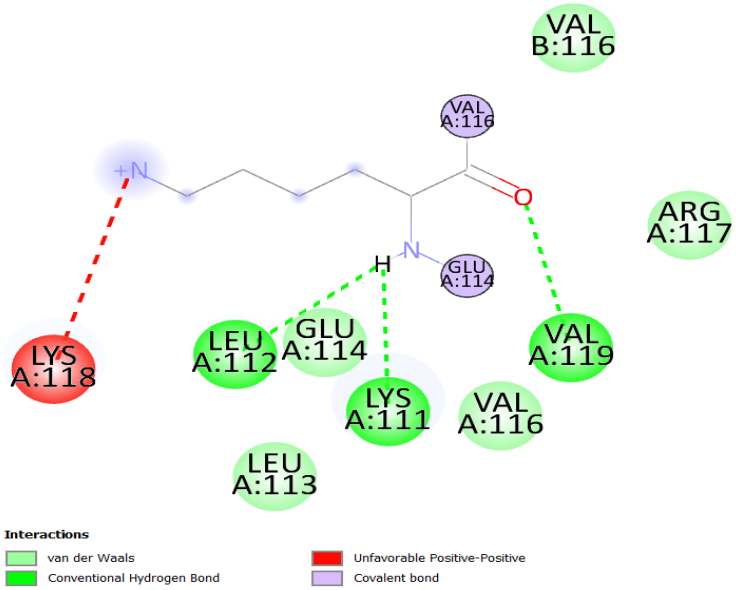


FIGURE 31: 2D complex of CAV-1 and Ligands interactions

6.3.5 INTERACTION BETWEEN TRANSFERRIN AND LIGANDS

The transferrins are a family of iron-binding proteins, which has a high affinity to ferric iron. Transferrin is a blood-plasma glycoprotein, which plays a central role in iron metabolism and ferric-ion delivery. The interactions formed between the selected twenty four ligands and Transferrin. The 2D and 3D structures were visualized using BIOVIA Discovery studio visualizer tool. The ligands were bonded to proteins with five van der Waals interaction such as ALA C:311, MET C:313, TYR C:314, LEU C:315, VAL C:320 and with one attractive charge such as ASP C:310 and with one conventional hydrogen bond such as GLY C:316 and with one pi-alkyl bond such as TYR C:71 and with two covalent bond such as ALA C:311, MET C:313 was shown in the Figure (34). It was observed that the compound Annomuricin B CID: 44575650 shows a better interaction with the Transferrin protein with the binding energy of -9.8 kcal mol−1.

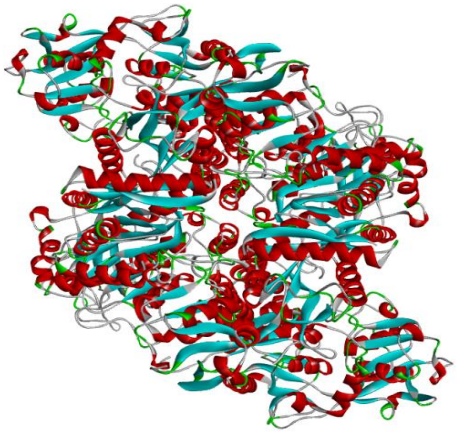


FIGURE 32: Structure of Transferrin Protein

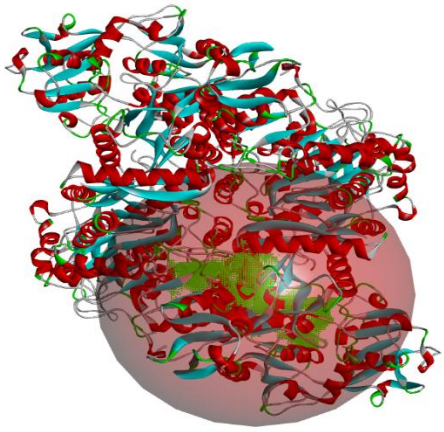


FIGURE 33: Binding site of Ligands with Transferrin

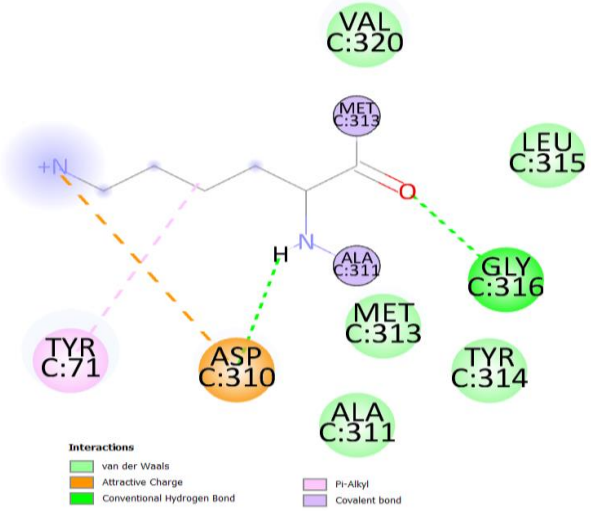


FIGURE 34: 2D complex of Transferrin and Ligands interactions

6.4 PREFORMULATION STUDIES

6.4.1 Physical Incompatibility studies:

Physical incompatibility study reveals that there is no change in color, odor and viscosity in the single and mixture of compounds.

6.4.2 Compatibility studies

FTIR is an extremely useful technique for confirming the identity of pure compounds by functional group determination. This FTIR spectrum shows that the functional groups in AM, DOX and Phospholipids are involved in the complex formation in AM-DOX liposome. AM showed the characteristic absorption regions at 3266.34cm^{-1}, 2928.28cm^{-1}, 1029.41cm^{-1} which are the assigned functional group –OH, C-H, C-O stretching respectively, DOX showed the characteristic absorption regions at 3315.81cm^{-1}, 3525.49cm^{-1}, 2895.31cm^{-1}, 1729.14cm^{-1}, 1412.33cm^{-1}, 1070.37cm^{-1} and 1579.11cm^{-1} which are the assigned functional group –OH, N-H,C-H, C=O, C-O, and C=C stretching respectively and Phospholipid showed the characteristic absorption regions at 2921.19cm^{-1}, 1734.05cm^{-1}, 1259cm^{-1} and 1073.90cm^{-1} which are the assigned functional group C-H, C=O, P=O and P-O-C stretching, respectively. The absorption peak of AM-DOX liposome spectrum was the sum of the characteristic AM and DOX peaks, the phenolic –OH peaks (3312.68cm^{-1}) of mixture spectrum were increased when compared to AM peak and were decreased when compared to DOX peak. In addition, the peak of C=O region of DOX and Phospholipid near 1728.82cm^{-1} shifted to little bit higher wavelength. And also their hydrogen bonding counterparts, such as P=O peak 1233.39$cm^{-1}$of P were increased and P-O-C peak signal near to 1070.14cm-1 in the AM-DOX liposome spectrum. These changes indicates the involvement of –OH, C=O, C-H, N-H, P=O and P-O-C groups in complex formation. Finally the FTIR peak of AM-DOX liposome was compactible to the individual compounds.

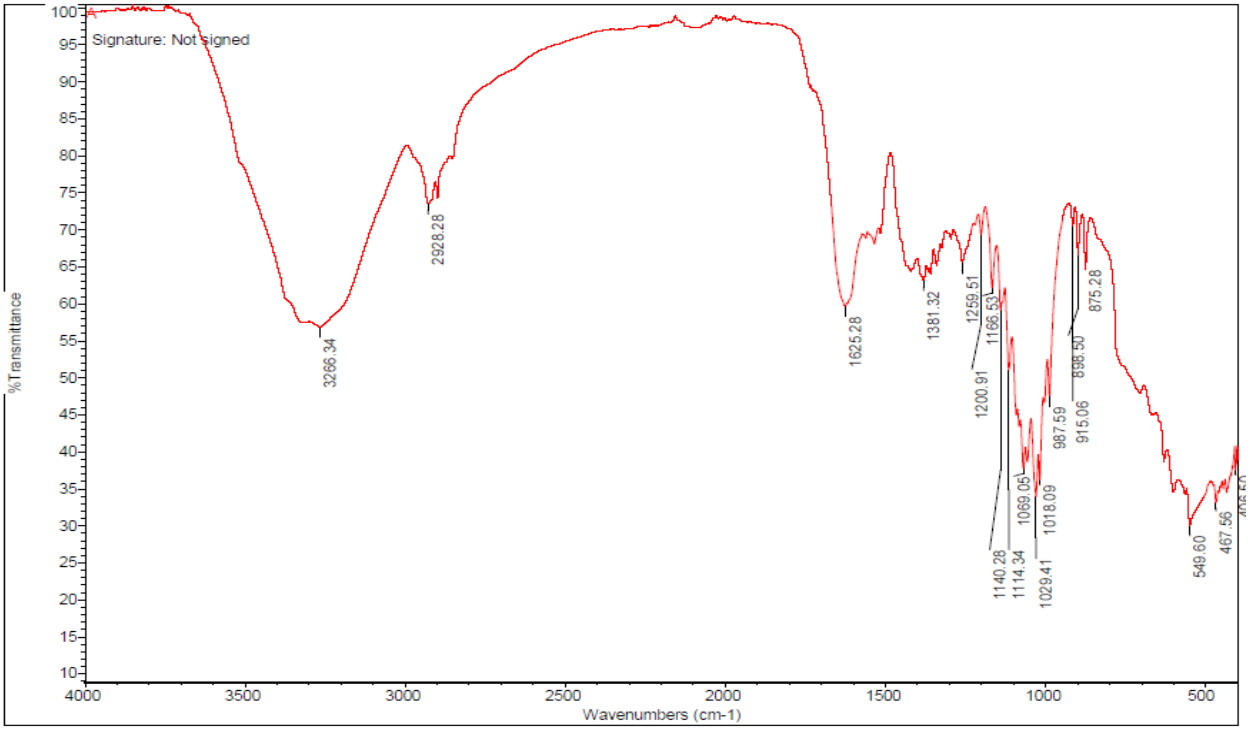


FIGURE 35: FTIR Spectrum of *Annona muricata*

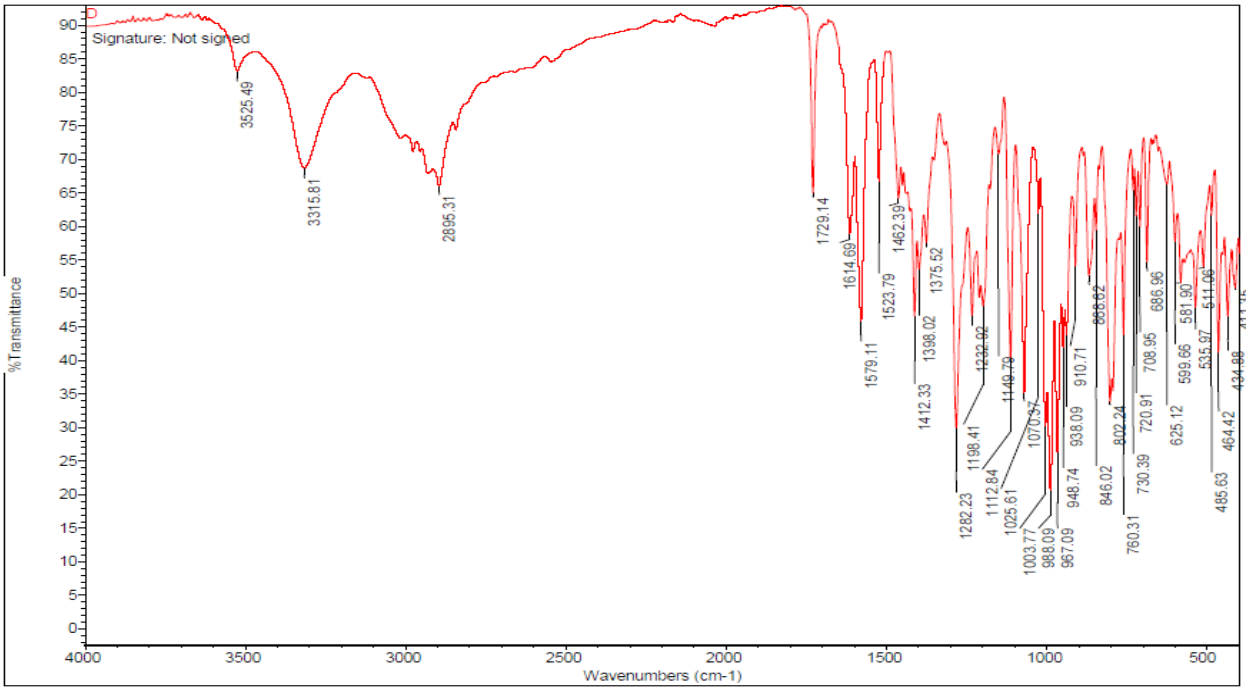


FIGURE 36: FTIR Spectrum of Doxorubicin

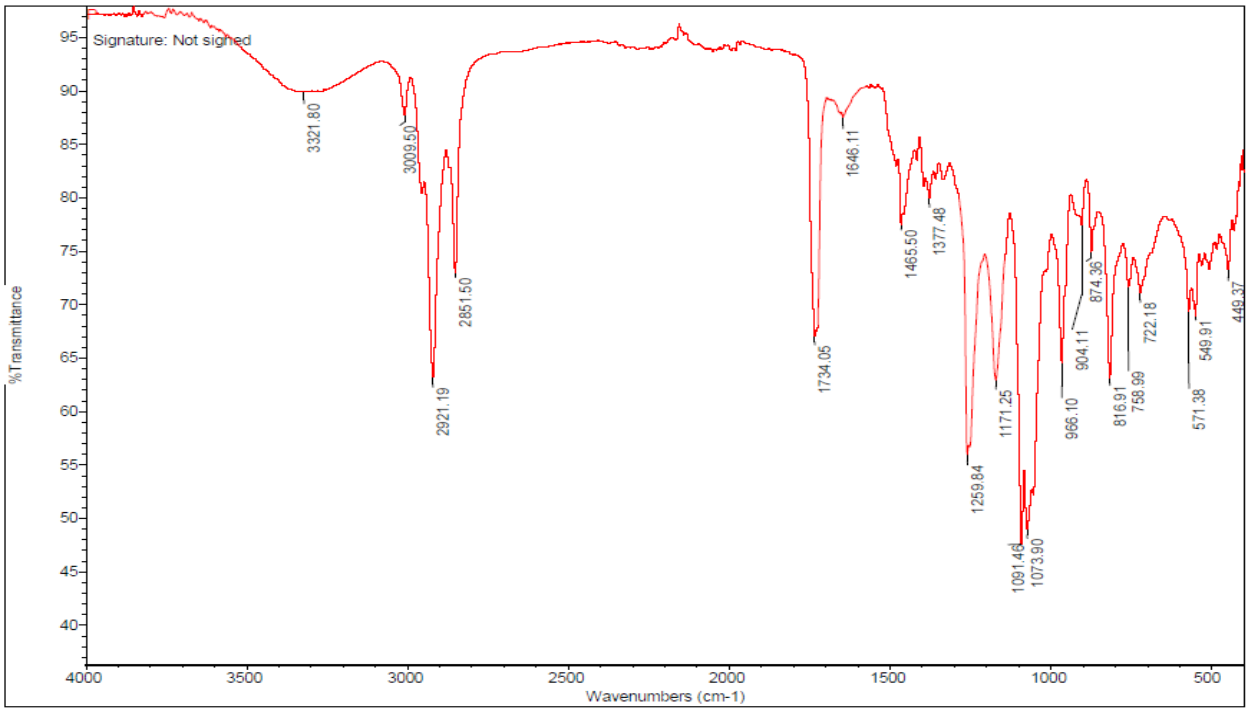


FIGURE 37: FTIR Spectrum of Phospholipid

FIGURE 38: FTIR Spectrum of AM-DOX Liposome

6.5 LIPOSOMAL CHARACTERIZATION AND EVALUATION

6.5.1 Physiochemical Characterization

Liposome formulations was prepared by thin film hydration method by using different drug mixtures. The different formulations have been shown in Table (18). The prepared AM, DOX and AM-DOX loaded liposomes has been shown in Figure (39). The liposomes were prepared with HSPC, DSPE-mPEG-2000 and CHO. AM, DOX, AM-DOX liposomes were made with lowest particle size and highest entrapment efficiency. The formulations AM, DOX and AM-DOX had a Particle size of 160 nm 147 nm and 181 nm; Poly dispersity index of 0.435, 0.215 and 0.625; Zeta potential of -10.7 mV, +0.774 mV and -6.87 mV respectively. Particle size, zeta potential and Poly dispersity index data of different liposomal formulations are shown in Table (18).

FIGURE 39: AM, DOX and AM-DOX Liposomes

TABLE 18: Physiochemical Characteristics of Liposomes

S.no	Formulation	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
1.	AM Liposome	160	0.435	-10.7
2.	DOX Liposome	147	0.215	0.774
3.	AM-DOX Liposome	181	0.625	-6.87

			Size (d.nm):	% Intensity:	St Dev (d.n..
Z-Average (d.nm):	160.0	Peak 1:	153.0	100.0	28.81
PdI:	0.435	Peak 2:	0.000	0.0	0.000
Intercept:	1.00	Peak 3:	0.000	0.0	0.000
Result quality:	Refer to quality report				

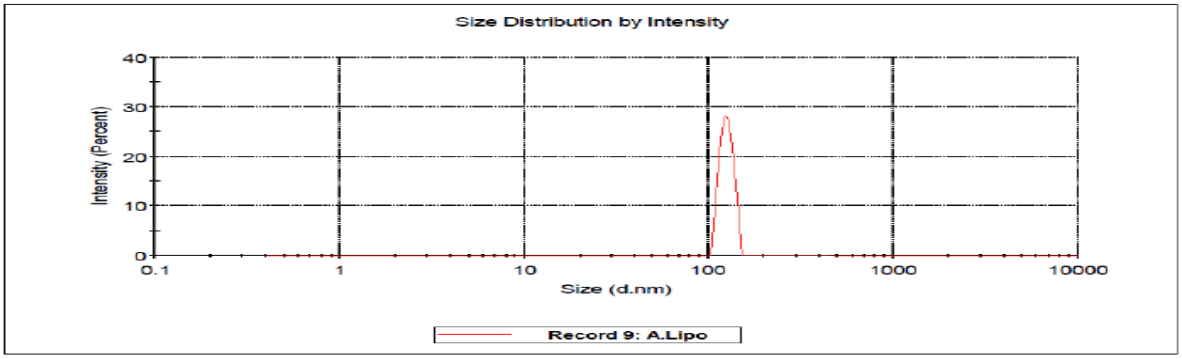


FIGURE 40: Particle size and PDI of AM Liposome

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	147.0	Peak 1:	121.8	100.0	38.81s
PdI:	0.215	Peak 2:	0.000	0.0	0.000
Intercept:	1.00	Peak 3:	0.000	0.0	0.000
Result quality:	Refer to quality report				

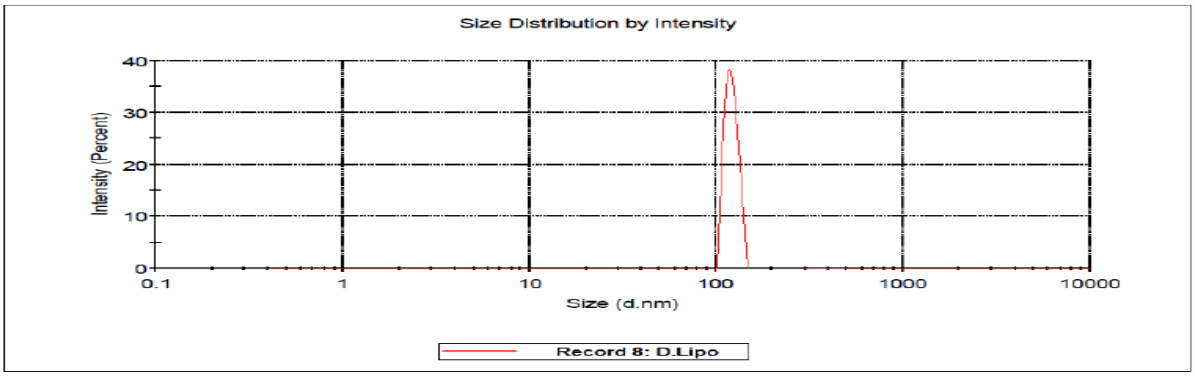


FIGURE 41: Particle size and PDI of DOX Liposome

			Size (d.nm):	% Intensity:	St Dev (d.n..
Z-Average (d.nm):	181.0	Peak 1:	163.8	100.0	32.81
PdI:	0.625	Peak 2:	0.000	0.0	0.000
Intercept:	1.00	Peak 3:	0.000	0.0	0.000
Result quality:	Refer to quality report				

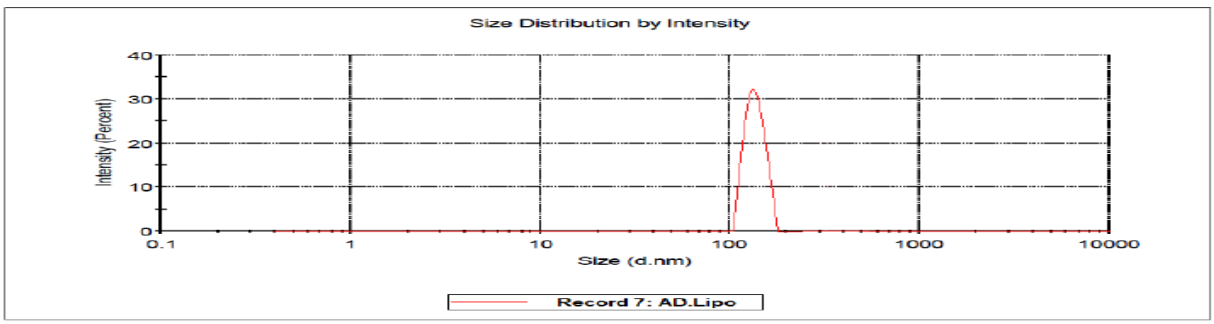


FIGURE 42: Particle size and PDI of AM-DOX Liposome

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -10.7	Peak 1:	-9.21	55.5	5.57
Zeta Deviation (mV): 43.7	Peak 2:	6.25	43.1	6.38
Conductivity (mS/cm): 0.0288	Peak 3:	105	1.0	0.701
Result quality : See result quality report				

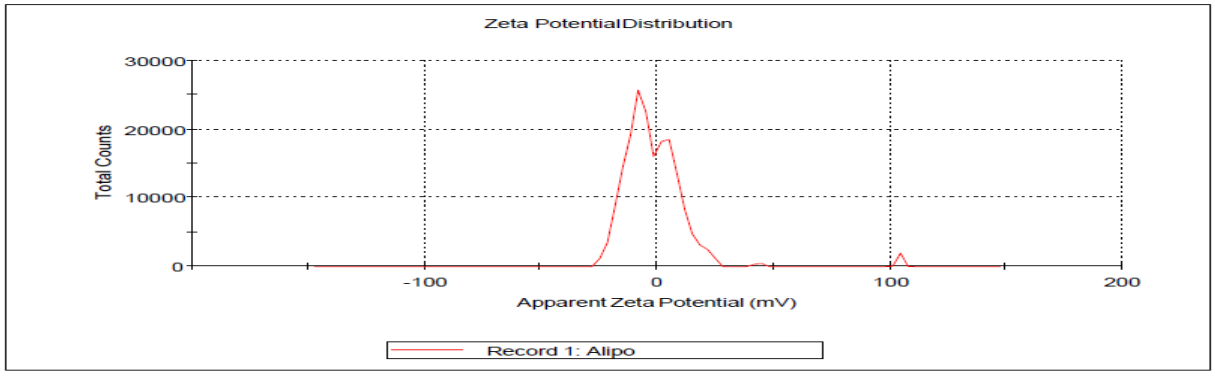


FIGURE 43: Zeta potential of AM Liposome

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): 0.774	Peak 1:	0.329	96.3	5.06
Zeta Deviation (mV): 5.71	Peak 2:	16.3	3.7	2.21
Conductivity (mS/cm): 0.252	Peak 3:	-22.8	0.0	3.37e-7
Result quality : Good				

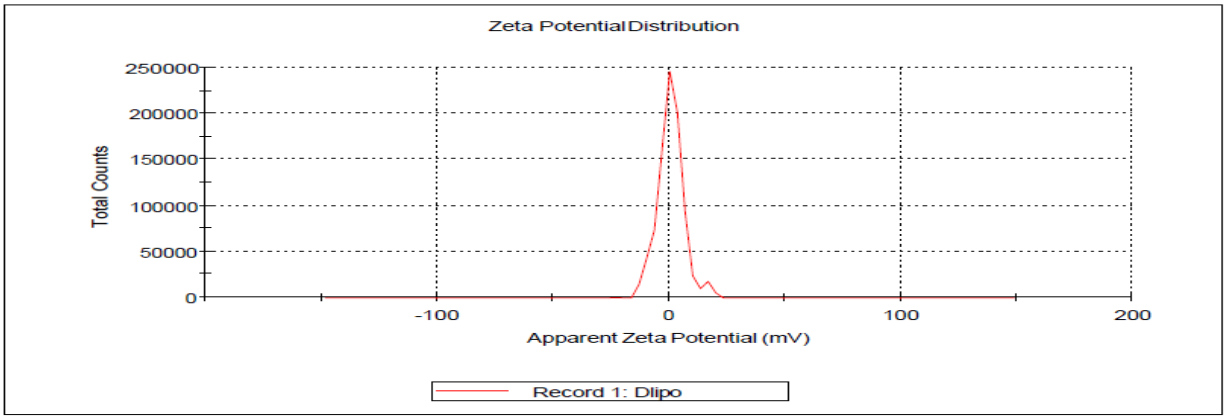


FIGURE 44: Zeta potential of DOX Liposome

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -6.87	Peak 1:	-6.87	100.0	5.43
Zeta Deviation (mV): 5.43	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0172	Peak 3:	0.00	0.0	0.00
Result quality : Good				

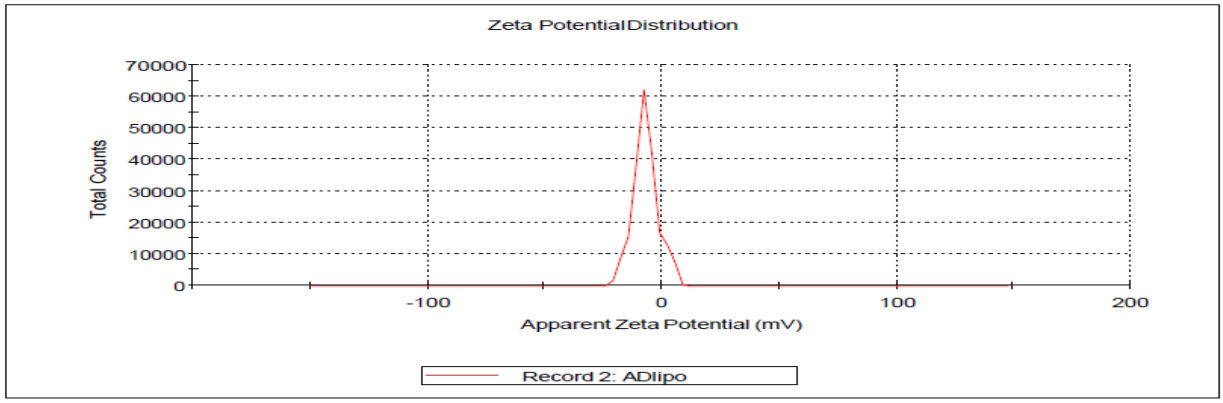


FIGURE 45: Zeta potential of AM-DOX Liposome

6.5.2 Entrapment Efficiency

The amount of entrapped drug was determined spectrophotometrically at 217 and 496 nm was shown in the Table (19) and Figure (46, 47). The calibration curve was linear in the range of 10–10,000 ng/ml. The absorbance was calculated using the formula y = mx+c, and the entrapment efficiency was calculated by using following formula (EE) = (CT−CS)/CT×100. Entrapment efficiency of AM, DOX and AM-DOX Liposomes are shown in the Table (19).

TABLE 19: Entrapment Efficiency of Liposomes

S.no	Formulation	Entrapment efficiency EE (%)
1.	AM Liposome	83.1
2.	DOX Liposome	86.6
3.	AM-DOX Liposome	84.85

TABLE 20: Absorbance value of DOX and AM

S.no	Concentration (µL)	Doxorubicin Absorbance (496nm)	Annona muricata Absorbance (217nm)
1.	2 µL	0.027	0.153
2.	4 µL	0.055	0.251
3.	6 µL	0.096	0.437
4.	8 µL	0.130	0.448
5.	10 µL	0.163	0.527

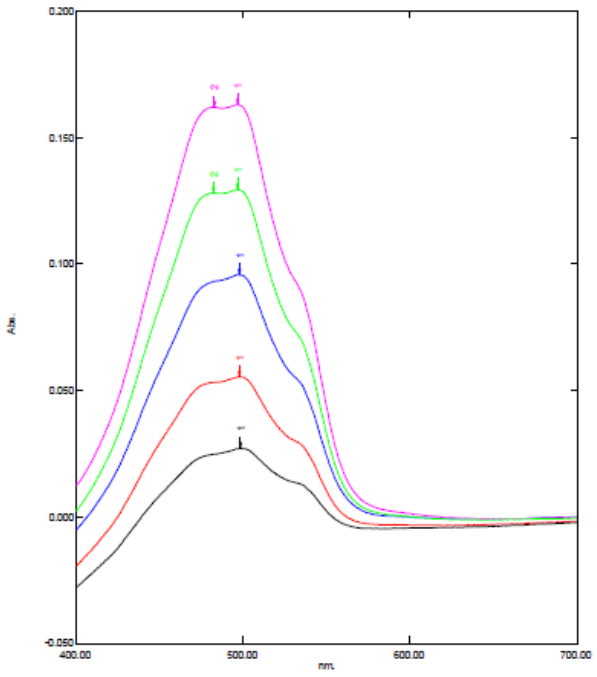


FIGURE 46: UV absorbance of DOX at 496nm

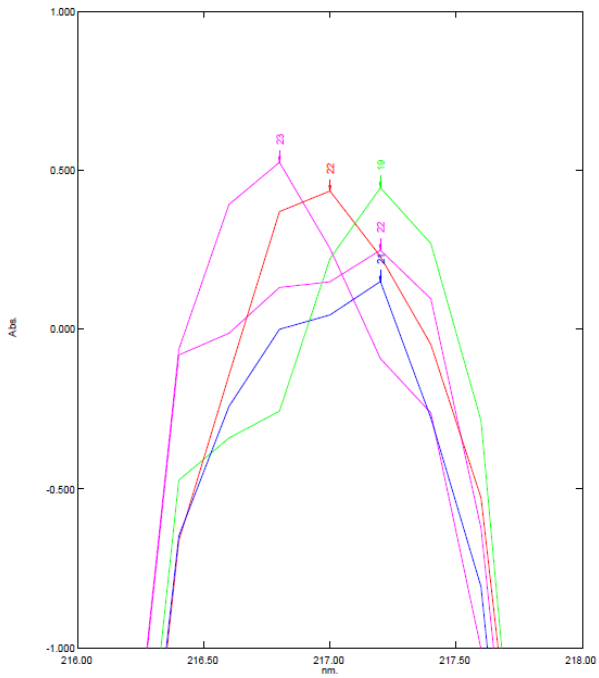


FIGURE 47: UV absorbance of AM at 217nm

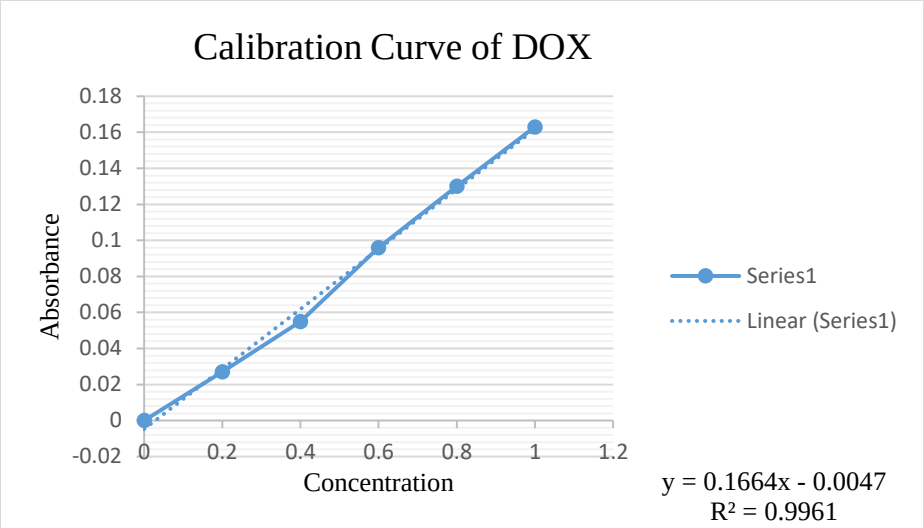


FIGURE 48: Calibration Curve of DOX

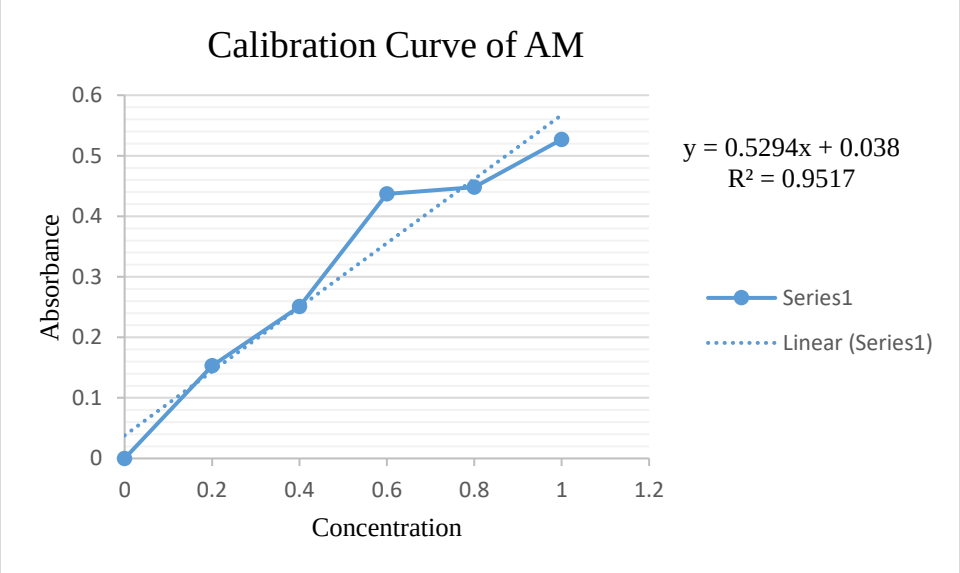


FIGURE 49: Calibration Curve of AM

6.5.3 Morphology

Liposomes were placed on copper grid films and stained with 2% (w/v) phosphotungstic acid for morphological observation by transmission electron microscopy (TEM) (JEM-100CX, JEOL, Japan). The transmission electron microscopy (TEM) images of the liposomes are shown in Figure (50). The liposomes look like vesicles, which are spherical in shape and have a diameter of about 140–200 nm.

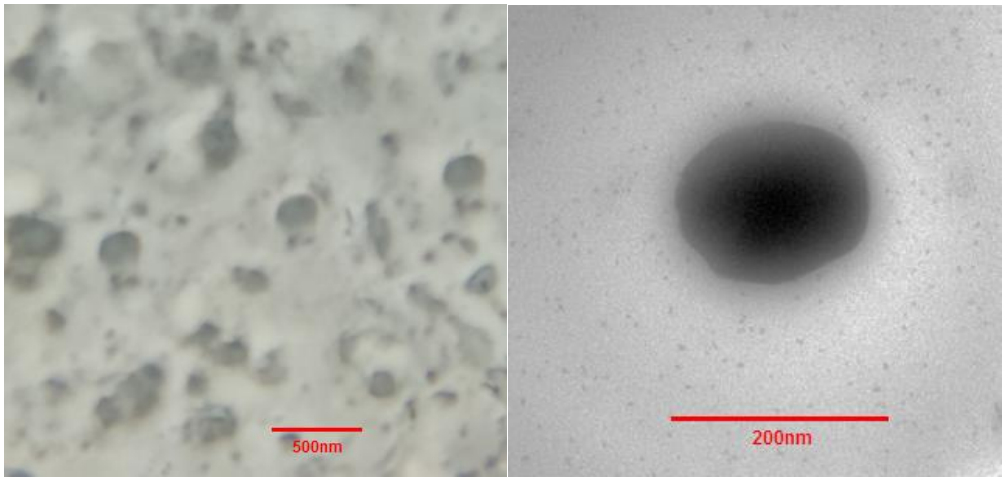


FIGURE 50: TEM Image of AM-DOX Liposome

6.5.4 Physical Stability of Liposomes

The stability problem of liposomes were limited to two aspects: the physical stability against aggregation or fusion of the dispersion and the drug retention (or latency), defined as the percentage of drug that is liposome-associated. The physical stability of formulations such as AM, DOX and AM-DOX liposomes at a storage of 2-8^{0}C are 2-3, 2-3, 1-2 weeks and then precipitates.

TABLE 21: Stability Study of Liposomes

S.no	Formulation	Physical Stability at 2-8^{0}C
1.	AM Liposome	Stable for 2-3 weeks, then precipitates
2.	DOX Liposome	Stable for 2-3 weeks, then precipitates
3.	AM-DOX Liposome	Stable for 1-2 weeks, then precipitates

6.4.5 Cytotoxicity of Liposomes on MDA-MB-231 Breast Cancer Cell line

The susceptibility of MDA-MB-231 Breast cancer cell lines were tested over the different formulations of liposomes such as AM liposome, DOX liposome and AM+DOX conjugated liposome using the standard cell viability test, the MTT assay. The assay proved that the cytotoxic effect of AM+DOX liposome was higher than that of AM liposome and DOX liposome. The formulation AM+DOX liposome shows the least cell viability of 33.1% at 100µg/ml concentration whereas AM liposome and DOX liposome shows the cell viability of 53.76% and 47.8% at 100µg/ml concentration respectively, which shows that the formulation AM+DOX liposome shows the highest percentage inhibition of MDA-MB-231 cell line was shown in the Table (22). The AM+DOX liposome shows the IC_{50} value of 4.672 µM which is lower than the IC_{50} value of AM liposome (6.109 µM) and DOX liposome (5.732 µM) was shown in a Figure (56). The AM-DOX liposomal drug delivery system significantly enhanced the delivery of *Annona muricata* leaf extract and Doxorubicin to the breast cancer cells and shows the least cell viability.

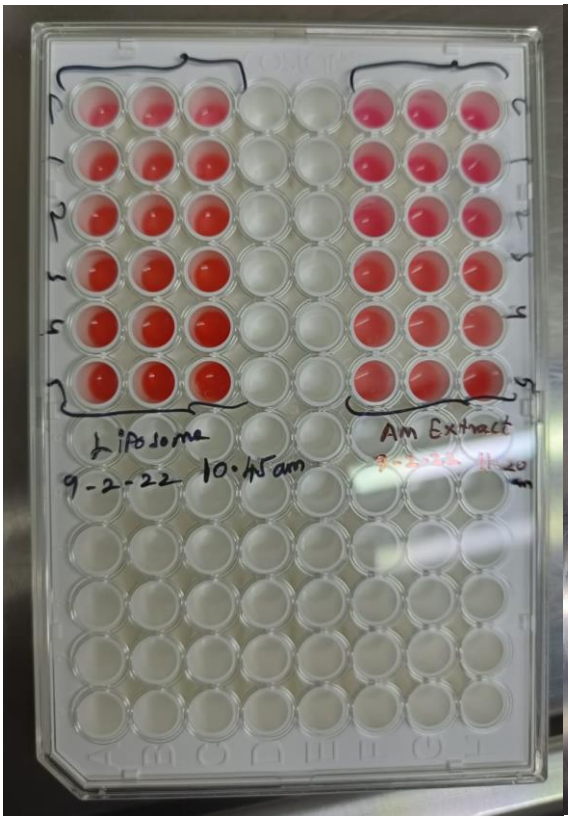


FIGURE 51: MDA-MB-231 Cell line Drug treated

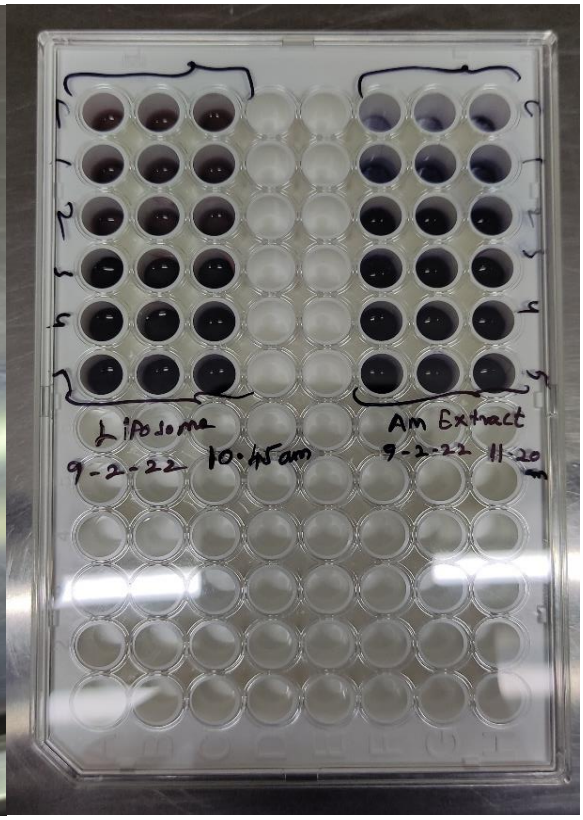


FIGURE 52: MDA-MB-231 Cell line MTT treated

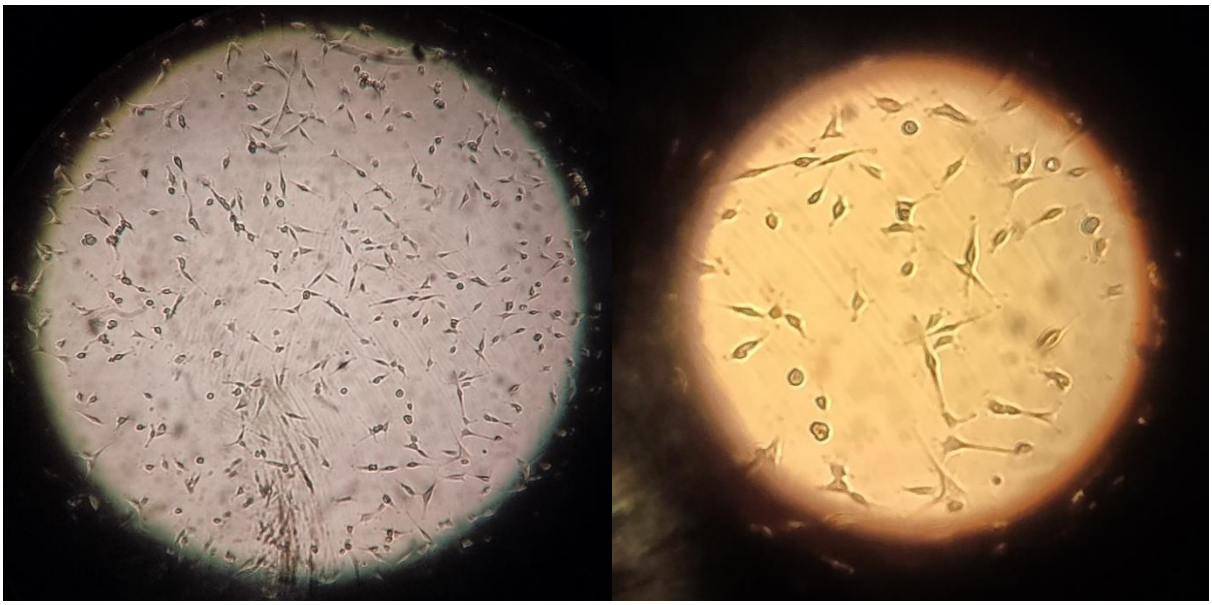
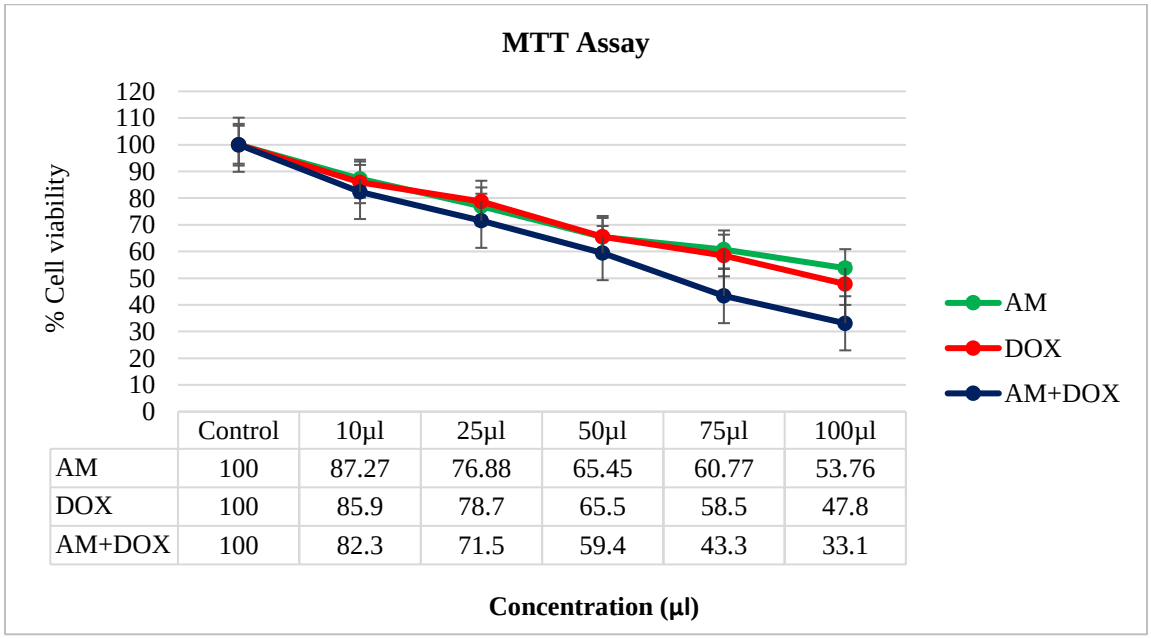


FIGURE 53: MDA-MB-231 Cell line before treatment FIGURE 54: MDA-MB-231 Cell line after treatment

TABLE 22: Percentage Cell viability of MTT Assay

Formulations	Control	10µl	25µl	50µl	75µl	100µl
AM liposome	100%	87.27%	76.88%	65.45%	60.77%	53.76%
DOX liposome	100%	85.9%	78.7%	65.5%	58.5%	47.8%
AM+DOX liposome	100%	82.3%	71.5%	59.4%	43.3%	33.1%



	Control	10µl	25µl	50µl	75µl	100µl
AM	100	87.27	76.88	65.45	60.77	53.76
DOX	100	85.9	78.7	65.5	58.5	47.8
AM+DOX	100	82.3	71.5	59.4	43.3	33.1

FIGURE 55: Percentage Cell viability of MTT Assay

TABLE 23: IC_{50} values for MTT assay using GraphPad Prism 9 software

log(inhibitor) vs. normalized response -- Variable slope	AM	DOX	AM+DOX
Best-fit values			
$LogIC_{50}$	1.890	1.576	2.777
Hill Slope	-0.8250	-1.388	-0.9597
IC_{50}	**6.109**	**5.732**	**4.672**
95% CI (profile likelihood)			
$LogIC_{50}$	73.94 to 178.8	(Very wide)	52.22 to 89.37
Hill Slope	-0.01608 to -0.003412	(Very wide)	-0.01972 to -0.007045
IC_{50}	8.753e+073 to 6.223e+178	(Very wide)	1.647e+052 to 2.346e+089
Goodness of Fit			
Degrees of Freedom	4	4	4
R squared	0.7716	0.9860	0.9859
Sum of Squares	346.8	25.65	43.42
Sy.x	13.17	3.518	4.695
Constraints			
IC_{50}	IC50>0	IC50>0	IC50>0
Number of points			
# of X values	6	6	6
# Y values analyzed	6	6	6

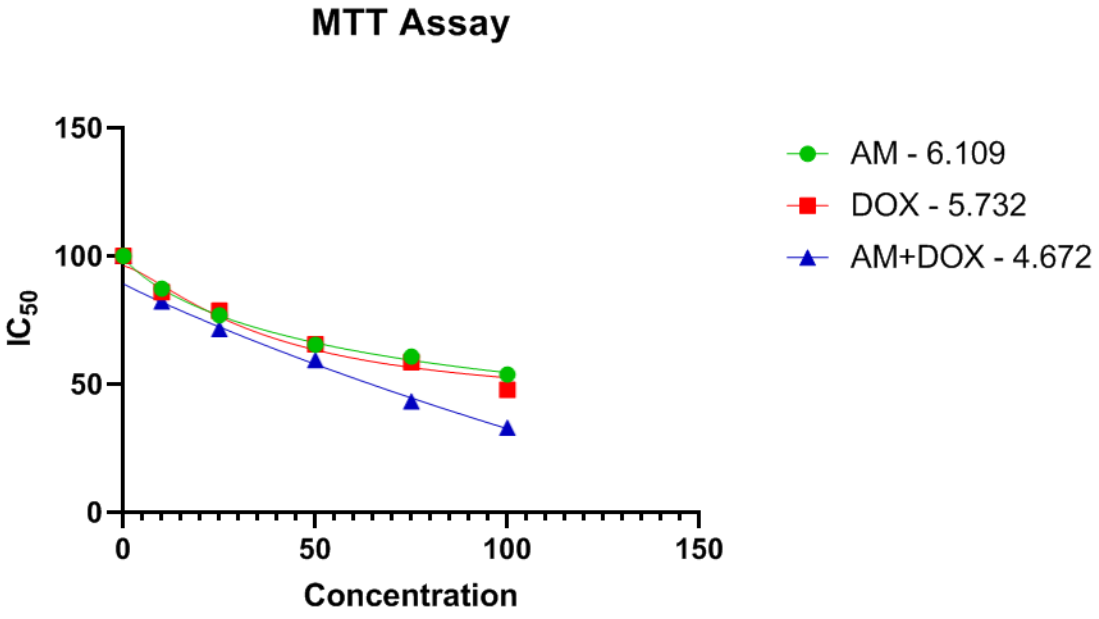


FIGURE 56: IC_{50} values of MTT assay

The *p*-value for the dose parameter is less than *p*-value 0.05, then the result is considered as significant. Hence there is a significant change in the viability of cells with the change in the dose. The *p*-value for the formulations is greater than *p*-value 0.05, then the result is not statistically significant. Hence the change in various formulations do not lead to a significant difference in the viability of cells. The *p*-value for the interaction effect of formulations and dose is less than *p*-value 0.05, hence we can conclude that there is a significant difference in the viability of cells with the combined effect of formulation and various dose. The estimated parameters are shown in the Table (24).

TABLE 24: Formulations and Dosage Parameter Estimation by 2-way ANOVA

Parameter Estimates							
			95% Wald Confidence Interval		Hypothesis Test		
Parameter	B	Std. Error	Lower	Upper	Wald Chi-Square	Df	Sig.
(Intercept)	96.740	2.8698	91.115	102.364	1136.319	1	.000
Formulations * Dose	-2.021	0.4388	-2.881	-1.161	21.215	1	.000
Formulations	0.508	1.3285	-2.095	3.112	0.146	1	.702
Dose	-6.835	.9479	-8.693	-4.978	52.004	1	.000

Dependent Variable: Response

Model: (Intercept), Formulations * Dose, Formulations, Dose

The *p*-value less than ($^{*}p<0.05$) were considered as significant.

7. CONCLUSION

This study aims on *in-silico* and *in-vitro* evaluation of breast cancer activity. The *in-silico* study focused against the P-glycoprotein, which hammers the therapeutic efficacy of various chemotherapeutic agents because of P-gp efflux mechanism. The present study involves in the collection of phytoconstituents of *Annona muricata* leaf and evaluated against the selected targets (MDR 1, ABCB1, AP2, Cav-1, Transferrin). *In-silico* study involves in the analysis of drug-likeness, ADMET analysis and molecular docking study.

- The drug-likeness study results reveals that almost all the Phytoconstituents of Annona muricata were not suitable for oral administration due to poor bioavailability and molecular weight more than 500 Daltons and all the ligands violates the two or more violations in Lipinski rule of five.

- ADMET analysis of the Phytoconstituents shows the significant results. Among the compounds Annocatalin, Annomuricin A, Annomuricin B, Annomuricin C, Annomuricin-D-one, Annomuricin E, Annomutacin, Annopentocin A, Annopentocin B, Annopentocin C, Iso-Annonacin, Muricatocin B and Muricatocin C exhibits the good ADMET profile.

- The present study shows that DOX can deliver into cancer cell by the inhibition of P-gp efflux by phytoconstituents present in *Annona muricata.* So, the therapeutic efficacy of DOX can be enhanced.

- Molecular docking of phytoconstituents reveals that Annopentocin A shows significant binding energy of (-9.2 kcal mol−1) with MDR 1 protein, Annocatalin shows significant binding energy of (-10.5 kcal mol−1), (-10 kcal mol−1) and (-7.6 kcal mol−1) with ABCB1, AP2 and CAV-1 protein and Annomuricin B shows significant binding energy of (-9.8 kcal mol−1) with Transferrin.

- The Physiochemical characterization of AM-DOX liposome shows the Particle size of 181nm; Polydispersity index of 0.625; Zeta potential of -6.87mV and Entrapment efficiency of 84.85%. Further this formulation is evaluated by cytotoxicity study using MDA-MB-231

breast cancer cell line. The formulation AM-DOX liposome shows the least cell viability of 33.1% at 100µg/ml concentration compare with AM liposome and DOX liposome shows the cell viability of 53.76% and 47.8% at 100µg/ml concentration respectively, which shows that the formulation AM+DOX liposome shows the more significant percentage inhibition of MDA-MB-231 cell line. The AM-DOX conjugated liposomal drug delivery system significantly enhanced the delivery of *Annona muricata* leaf extract and Doxorubicin to the breast cancer cells and shows the least cell viability.

FUTURISTIC STUDY

- *In-silico* study shows only predicted values but scientific validation of these ligands requires further *in-vitro* and *in-vivo* animal studies.

- Further, these studies to be carried out in future by isolation of particular above mentioned compounds from *Annona muricata* and making the novel drug delivery formulation to overcome the P-gp efflux by inhibiting the multi-drug resistance proteins in the breast cancer to promote the therapeutic efficacy of the chemotherapeutic agent.

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ANNEXURE

भारतसरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA

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सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2021/Tech. /301 दिनांक/Date: 6th December 2021

पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Annona muricata* L. - ANNONACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

डॉ. एम. यु. शरीफ़/DR. M. U. SHARIEF
वैज्ञानिक 'ई' एवं कार्यालयाध्यक्ष/
SCIENTIST 'E' & HEAD OF OFFICE

सेवा में / To

Mr. PRAVEEN KUMAR K N
Final Year M.Pharm. Student
Department of Pharmacology
Karpagam College of Pharmacy
COIMBATORE – 641 032

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K.N.Praveen Kumar

has participated in the International webinar on **"Recent Advances in Preclinical Research"** organized by Department of Pharmacology, Chalapathi Institute of Pharmaceutical Sciences, Guntur, Andhra Pradesh, INDIA in association with SERB (Science and Engineering Research Board), held on **06th JUNE 2020.**

Sri.Y.V Anjaneyulu
President
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Prof.Rama Rao Nadendla
Principal
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ARISE Online National Faculty Development Programme on Latest Trends in Pharmacology

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Certificate of Participation

This is to certify that

Mr. K.N.PRAVEEN KUMAR

KARPAGAM COLLEGE OF PHARMACY

has participated in the Online National Faculty Development Programme

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