EXPLORATION OF ANTICANCER POTENTIAL OF POTENT, NATURAL PPARγ AGONIST AGAINST TRIPLE NEGATIVE BREAST CANCER BY IN SILICO, IN VITRO AND IN VIVO METHODS

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

In

Biochemistry

By

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LOVELY PROFESSIONAL UNIVERSITY, PUNJAB 2024

DECLARATION

I, hereby declared that the presented work in the thesis entitled "**Exploration of anticancerpotential of potent, natural PPARγ agonist against Triple negative breast cancer by in silico, in vitro and in vivo methods**" in fulfilment of degree of **Doctor of Philosophy (Ph.D.)** is outcome of research work carried out by me under the supervision of Dr. Jeena Gupta, working as associate professor, in the School of Bioengineering & Biosciences of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "**Exploration of anticancer potential of potent, natural PPARγ agonist against Triple negative breast cancer by in silico, in vitro and in vivo methods**" submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the department of Biochemistry,School of Bioengineering & Biosciences, is a research work carried out by Aishwarya Laxmi, 11719236, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

Breast cancer is characterized by uncontrolled growth of abnormal breast cells. It is caused worldwide mostly due to inheritance of mutated genes like BRCA1 and BRCA2. However, other factors like reproductive history, female gender and longer exposure of women to hormones (like starting menstrual age before 12 and ending after age of 55) increases risk of breast cancer. However, Triple negative breast cancer (TNBC) differs from other types of breast cancers in some of the risk factors like hormonal exposure because in TNBC hormone receptors for estrogen, progesterone and Her2 neu are absent. So, hormonal significance doesn't matter in anyway, thus TNBC patients don't get benefit of hormonal treatment. So, due to lack of hormone receptors in TNBC, treatment options remains very limited for patients. Another difference is inheritance of faulty BRCA1 gene in TNBC patients. Mostly TNBC patients have mutated BRCA1 gene. BRCA1 gene mutation increases susceptibility of body's cells for further genetic alterations leading to advancement in TNBC. TNBC is an aggressive cancer due to frequent metastasis, high recurrence rate and very low survival rate in comparision to other types of breast cancers. Current treatment options like lumpectomy, mastectomy, chemotherapy and radiotherapy are there as treatment options but there are several side effects associated with these therapeutic strategies like hair loss, nausea, tiredness, lymphedema, skin changes etc. So, to avoid these side effects some other potent treatment strategy need to be discovered.

Alternative treatment strategy may include consideration of other receptor which is expressed in TNBC cells. One such expressed receptor in TNBC cells is PPAR-γ receptor, which is a nuclear hormone receptor. However, PPAR-γ is, already highly explored for treatment of diabetes melitus but still very less explored receptor for TNBC. Thus, there is need to explore this receptor for treating TNBC. PPAR-γ after activation by its agonists creates hostile environment for growth of TNBC cells. PPAR-γ is found involved in decreasing proliferation of differet types of cancer cells including breast cancer. It also increases apoptosis through different pathways like Akt/mTOR etc, by activating different proteins like E-Cadherin etc. Activation of PPAR-γ by synthetic agonists like thiazolidinediones (TZDs) for eg. Troglitazone, Pioglitazone and Rosiglitazone is also associated with serious side effects like edema, heart attack, hypertension, myocardial ischemia, upper respiratory tract infection, diarrhea etc, thus due to these side effects one of the drug rosiglitazone became banned in US, for its usage along with insulin against the treatment of diabetes mellitus. Thus, these synthetic PPAR-γ agonists had not proved themselves as good agonists due several risky side effects,

however they were activating PPAR-γ fully. Since these synthetic thiazolidinediones were chemicals, so they were showing so deadly side effects. Thus, problem can be solved if PPAR-γ can be activated by natural PPAR-γ agonists, instead of synthetic ones. NaturalPPAR-γ agonists are phytochemicals which activates PPAR-γ receptor partially, targeting particular gene only.

Thus, natural PPAR-γ agonists results in less or no side effects which was actually the demand of therapeutic realm of TNBC.

In current research, we performed insilico, invitro and invivo experiments to explore natural, potent PPAR-γ agonists having anticancer potential against TNBC. For this firstly in insilico study, natural phytochemical PPAR-γ agonists were selected from literature for similarity search in PubChem database. Searched compounds were undergone through docking process in Autodock Vina to confirm PPAR-γ agonistic activity. Further compounds having highest dockscore underwent ADME study in 5 different softwares namely SwissADME, vNNADMET, ADMETlab, admetSAR and DruLiTo considering 17 different parameters out of all different parameters in result. Finally, 3 compounds were found as good potent compounds having drug like activity. These three compounds undergone molecular dynamics study in Desmond of Schroedinger suite to see molecular interactions in dynamic biological environment to study exact drug like capabilities of compounds.

In invitro analysis, silymarin and hesperidin were explored for antioxidant, antiviability, anticancer potential. They were tested invitro for their PPAR-γ agonistic activity, their ability to assist in PPAR- γ expression. All these objectives were achieved by performimg different types of invitro assays like DPPH free radical scavenging assay, MTT assay, antioxidant enzyme activities assay like catalase test, glutathione s transferase test etc, Western blotting, Real time PCR and transcription factor binding assay. Both Silymarin and hesperidin were tested in DPPH assay to assess their antioxidant potential. Further, both the compounds underwent cell viability assay, in MDA MB-231 cells which are TNBC cells of human origin. So, we found that both the compounds were killing cancer cells with increasing concentration but hesperidin was having better result than silymarin w.r.t. doxorubicin, a standard drug for TNBC. Whereas, when both compounds were tested on L929 cells, mouse normal skin cells then it was observed that both the compounds silymarin and hesperidin kill very less normal skin cells in comparision to doxorubicin which showsthat both natural compounds were not toxic for normal cells but had toxicity only for cancer cells. Further, invitro study included antioxidant

enzyme activity test, in which both silymarin andhesperidin showed good antioxidant potential as it reduces oxidative stress by increasing activity of enzymes like Superoxide dismutase, catalase, glutathione s transferase as both compounds areherbal compounds, however doxorubicin being chemical compound does not increases activity of these enzymes in cancer cells and it simultaneously increases lipid peroxidation suggesting that it increases oxidative stress overall. Whereas, silymarin and hesperidin does not increase lipid peroxidation being phytochemicals, thus this shows that both silymarin and hesperidin has good antioxidant potential. For assessing PPAR-γ agonistic activity of these compounds, Transcription factor binding assay was performed using pioglitazone as standard compound. In this assay we found that Pioglitazone being synthetic PPAR-γ agonist had highest PPAR-γ agonistic activity. After it, hesperidin had better activity than silymarin; doxorubicin being synthetic chemical compound had least activity. In real time PCR, we assessed expression of certain genes like PPAR- γ, BRCA1, p53, SDC1, BCL2 and BAX expressed in cancer cells after its treatment with both the compounds silymarin and hesperidin. These genes were involved in progression and combating of cancer. we found that after treatment with both the compounds genes involved in combating cancer, acting as tumor suppressor were upregulated whereas genes involved in progression of cancer were down regulated. Thus, this shows that both compounds acted as anticancer compounds. Further, we performed western blotting too to assess the expression of PPAR-γ receptor in presence of both silymarin and hesperidin. We found that hesperidin causes better expression of PPAR-γ thansilymarin in MDA MB-231 cells.

Finally, we also performed invivo studies on fertilized egg, which included CAM angiogenesis assay and cell invasion assay. In CAM angiogenesis assay, we found that doxorubicin being anticancer chemotherapy standard drug completely inhibits formation of blood vessels in fertilized chicken egg whereas silymarin and hesperidin inhibits angiogenesis at higher concentration. However, angiogenesis inhibition was found more with hesperidin instead of silymarin. So, for finalassessment of antimetastatic potential of compounds, we chose hesperidin as it was found better than silymarin in angiogenesis assay. In cell invasion assay, we found that at 3 µg some MDA MB-231 cells penetrated germ layers of chicken egg whereas hesperidin at increased concentration completely inhibited metastasis of MDA MB-231 cells in chicken egg like doxorubicin. Thus, by all these results we had concluded that hesperidin has good anticancer properties and can act as potent therapeutic option against TNBC.

ACKNOWLEDGEMENT

In the name of Almighty God

वक्रतुण्ड महाकाय सूर्यकोटि समप्रभ |
निर्विघ्नं कुरु मे देव सर्वकार्येषु सर्वदा ||

All praises to the Almighty God who always strengthened my thoughts of moving forward towards completion of this research work and always fulfilled my heart with new hopes for newer opportunities. I can't express my love and gratitude in words to almighty for making me capable to overcome all odds in life and always blessing me with his love. During this research work there were several emotional downfalls with which I dealt courageously just because I felt God's gracious presence always with me which kept me going forward hopefully without worrying for results.

I acknowledge my heartfelt debt and express my very sincere thanks and gratitude to my respected and esteemed supervisor **Dr. Jeena Gupta Bansal**, Associate professor, Department of Biochemistry, School of Bioengineering and Biosciences, Lovely Professional University, for her scientific guidance, creative suggestions, constructive criticism, immense knowledge, attentive instructions, altruistic behavior and complete support in every respect for completion of this research work. It was my honour, pleasure and good luck to have her as my supervisor. I enjoyed every moment working with her.

I also want to extend my very sincere heartfelt thanks to my co-supervisor **Dr. Pawan kumar Gupta**, Associate professor, Department of Pharmaceutical Chemistry, Shri Vile Parle Kelavani Mandal's Institute of Pharmacy, for his creative guidance, support, vast knowledge, helpful suggestions and corrective criticism in my in silico work. It was my pleasure to work with him. I also acknowledge sincere help provided by **Dr. Sonam Bhatia** in completion of my in silico work, without her it would have never been possible. I am grateful to you mam from bottom of my heart.

I prolong my gratitude to **Prof. Dr. Neeta Raj Sharma**, Head of the School of Bioengineering and Bioscience, **Dr.** Head of the Department of Biochemistry, **Dr. Minhaz Ahmed**, Head of Laboratory School of Bioengineering and Bioscience and all other faculty members of the School of Bioengineering and Bioscience for providing all the facilities and for their full support toaccomplish my research work successfully.

I'd like to take this opportunity to thank **Mr. Ashok Mittal** (Chancellor), **Mrs. Rashmi Mittal** (Pro- Chancellor), **Dr. Preeti Bajaj** (Vice Chancellor), **Dr. Loviraj Gupta** (Executive Dean),

and **Dr. Monica Gulati** (Registrar) for establishing and maintaining such a dignified and distinguished university with comfortable work culture.

I also express my gratitude to our lab technicians, **Mr. Rahul Sharma, Mr. Kuldip, Mr. Sunny, Mr. Rajesh, Ms. Sandeep Kaur, and Mr. Madan**, who always helped me in fulfilling my lab work related requirements during my research.

It's impossible to forget untimely help and support given by my dear friends. I would like to express heartfelt thanks to **Nancy Bhura, Sourbh Garg**, **Kriti Kushwaha, Rupal Dubey, Sana ShafiBhat, Bhumandeep** and **Debojyoti Mandal** for all the good moments we shared during this entire journey of research work. I also extend heartfelt thanks to my dear friend **Fahad Arshad** for emotional encouragement and support given by him to complete this research work.

I dedicate this thesis to my family which is the reason I thrive to be better in life. I bow my head into the feets of my parents **Dr. Raj Kishore Mishra** and **Mrs. Urmila Mishra** for giving me this wonderful life and making me capable and confident to reach at this stage of life. I become speechless to pay my thanks and gratitude for their endless sacrifices and support to fulfill all my needs in life. I wholeheartedly thank my husband **Mr. Nidhi Kumar Awasthi,** for always believingin me so strongly and supporting me to fulfill all those dreams which were always so difficult for me. I would not have even thought of starting this research work without your support and encouragement. I can't express my emotions, thanks, love and gratitude in words for you. I can't forgetto pay my heartfelt sincere thanks and gratitude to my elder sister **Ms. Vijay Raj Laxmi** for her constant love and endless support without which it was impossible for me to even complete this research work and write thesis. I extend my love and thanks to my younger sister **Dr. Ravi Rani** forher support and care. I also want to thank my nephew **Saubhagya Shukla** for helping me in technical issues during my research and thesis compiling. Last but not the least, I definitely want to thank my two little, cute, naughty and highly demanding sons **Ritwik Awasthi** and **Ayaan Awasthi**, for whom I want whole world. Their sweet faces compelled me to complete this thesis and to move towards better prospects in life.

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LIST OF ABBREVIATIONS

S.No.	Abbreviations	Full form
1	IBC-NST	Invasisve Breast Carcinoma of no special type
2	ISDC	In situ ductal carcinoma
3	MSC	Mammary secretory carcinoma
4	FNAC	fine needle aspiration and cytology
5	MRI	Magnetic resonance imaging
6	BCT	breast conservation therapy
7	LRR	Locoregional recurrence
8	HR	hormone receptors
9	TNBC	triple negative breast cancers
10	WBI	whole breast irradiation
11	PMR	Post mastectomy radiation
12	NAC	Neoadjuvant chemotherapy
13	WBI	whole breast irradiation
14	PD-L1	Programmed death cell ligand 1
15	ER	estrogen receptor
16	PR	progesterone receptor
17	HER2 neu receptor	human epidermal growth factor receptor 2 neu
18	PPAR	Peroxisome Proliferated activated receptor
19	RXR	retinoid x receptor
20	PPARG	Peroxisome Proliferated activated receptor gamma
21	LBD	ligand binding domain

22	DBD	DNA binding domain
23	AF 2	Activation function 2
24	LPL	lipoprotein lipase
25	TZD	Thiazolidinediones
26	SLE	Systemic Lupus Erythematosus
27	CAF	cancer associated fibroblasts
28	CAA	cancer associated adipocytes
29	TME	tumor microenvironment
30	ECM	extracellular matrix
31	MCT-1	monocarboxylic lactate transporter-1
32	PDK	pyruvate dehydrogenase kinase
33	SLE	Systemic Lupus Erythematosus
34	MCT-1	monocarboxylic lactate transporter-1
35	TRAIL	TNF-associated apoptosis-inducing ligand
36	5-FU	5-fluorouracil
37	EGCG	epigallocatechin 3-gallate
38	EET	epoxy-eicosatrienoic acid
39	dLGG	di-*O*-α-linolenoyl-3-*O*-β-galactopyranosyl-*sn*-glycerol
40	CI/RI	Cerebral ischemia/ reperfusion injury
41	UUO	unilateral ureteral obstruction
42	RMSD	Root-mean-square deviation
43	ADMET	Adsorption Distribution Metabolism Excretion Toxicity
44	MDS	Molecular dynamics simulation

45	DPPH	1, 1 diphenyl-2-picrylhydrazyl
46	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
47	DMSO	Dimethyl sulfoxide
48	PDB	Protein data bank
49	BSA	bovine serum albumin
50	ELISA	Enzyme linked immuno sorbent assay
51	EDTA	Ethylenediamine tetraacetic acid
52	CDNB	1-Chloro -2-4, dinitrobenzene
53	GSH	reduced glutathione
54	GST	Glutathione s- transferase
55	DDW	Double distilled water
56	PUFA	Polyunsaturated fatty acids
57	MDA	Malondialdehyde
58	TBA	Thiobarbituric acid
59	TCA	Trichloroacetic acid
60	CAT	Catalase
61	O.D.	optical density
62	SOD	Superoxide dismutase
63	NBT	Nitroblue tetrazolium
64	SDS- PAGE	Sodium dodecyl sulphate- polyacrylamide gel Electrophoresis
65	TBS	Tris-Buffered saline
66	APS	Ammonium persulphate
67	TEMED	Tetra methyl ethylene diamine

68	RT	room temperature
69	TBST	tris buffer saline tween
70	ECL	enhanced chemiluminescence
71	PPRE	Peroxisome Proliferator response elements
72	PMSF	Phenylmethylsulphonyl fluoride
73	PCR	Polymerase chain reaction
74	PBS	Phosphate buffer saline
75	HRP	Horse reddish peroxidase
76	CAM	Chorioallantoic membrane
77	PDXs	Patient-derived xenografts
78	IC	Inhibitory concentration
79	WS	Working solution
80	ECM	Extracellular matrix proteins
81	DMEM	Dulbecco’s modified eagle medium
82	DPBS	Dulbecco’s Phosphate Buffered Saline
83	FBS	Fetal bovine serum
84	TBS	Tris Buffered Saline
85	QSAR	Quatitative structure activity relatioship
86	PPB	Plasma protein binding
87	HIA	human intestinal absorption
88	BBB	Blood brain barrier
89	VD	Volume distribution
90	DILI	Drug induced liver injury

91	QED	quantitative estimate of druglikeness
92	SPC	Single point charge
93	RMSF	root mean square fluctuation
94	LPO	Lipid peroxidation
95	ROS	Reactive oxygen species
96	DNA	Deoxyribo nucleic acid
97	TGZ	Thioglitazone

CHAPTER 1
INTRODUCTION

INTRODUCTION

1.1. Breast Cancer

Breast cancer (BC) is the cancer of breast tissue. It is the most commonly found cancer of females. Symptoms of breast cancer includes lump formation in breast tissue, dimple formation in skin, change in breast shape, inverted nipple, discharge of fluid from nipple, appearance of scaly red patches on skin (Boyd et al., 2007), (Watson M. et al, 2008), (Kollmorgen D.R. et al, 1998). Breast cancer is found as major cancer worldwide, thus considered as serious reason of death among Hispanic and Black women (Giaquinto et al., 2022) (Miller et al., 2021). Almost 30% cases of breastcancer are due to adjustable risk factors like physical inactivity, increased body weight and alcohol consumption etc., which can be prevented (Islami et al., 2018). However, few other non- modifiable risk factors include first menstruation at early age, radiation exposure, hormone replacement therapy and genetic factors etc. Breast cancer generally develops inside the lobules of breast which provide milk supply and cell lining for the milk duct. Breast cancer is usually diagnosed by biopsy of lumps of breast tissue, which is followed by treatment options like surgery, chemotherapy, radiotherapyand hormonal therapy (El-Bahr et.al, 2013). Chemotherapeutic drugs like tamoxifen are found to prevent breast cancer. Mastectomy is found to be best preventive measure of breast cancer. However, treatment of metastatic patient generally aims at improving the quality of their life (Crumley et al., 2013).

1.1.1. Epidemiology of BC

Breast cancer is most commonly occurring cancer after lung cancer in women of United States. 30% of new cancers in female are found to be breast cancer every year. In United States, risk of getting breast cancer is 13%, and rate of breast cancer incidence is increasing per year by 0.5%. American cancer society estimated 43,700 deaths and 55,720 novel cases of in situ ductal carcinoma, 297,790 novel cases of invasive breast cancer, will be diagnosed in 2023 in United States (Siegel et al, 2023). Breast cancer represents 11.7% of all cancer cases (Sung et al, 2021). Till year 2030, the burden of breast cancer is most likely to increase globally more than 2 million (Desantis et al, 2011). In India, breast cancer incidence is found to significantly increase by 50% from 1965 to 1985 (Saxena et al, 2002). According to Globocan in 2020, breast cancer represents 13.5% of all different types of cancers and 10.6% of different types of cancer deaths (Globocan et al, 2020).

Breast cancer especially occurs in middle and lower aged group females. 62 years is the median age for breast cancer diagnosis, which indicates thathalf of the affected patients gets diagnosed at 62 or younger age. Very few females are diagnosed at less than 45 years. Since 1989, death rate due to breast cancer is decreasing continuously as a result of early screening, more awareness and better treatments (Siegel et al, 2023). According to American Cancer Society screening for breast cancer must start at age of 40 for better diagnosis, treatment and survival (Smith et al, 2003).

1.1.2. Etiology of BC: (Abdulkareem et al, 2013)

There are different aetiological factors which can be explained under following headings.

- **Age**: It is observed by different observers that incidence of breast cancer increases with age. It is found that mostly cases use to be of more than 45 years of age. This shows the role of female reproductive hormones in pathogenesis of breast cancer as incidence is related with the exposure to hormone in reproductive life of female. Moreover, the age of menarche and menopause also matters. Early menarche and late menopause increases the duration of exposure of estrogen which has carcinogenic effect and helps in breast cancer development when collaborated with environmental and genetic factors.
- **Geography**: Females of western countries are more prone to get this disease in comparision to Indian females.
- **Gender**: Breast cancer is specially a disease of females, only less than 1% of breast cancer cases are males.
- **Diet and alcohol:** Excessive intake of alcohol and diet low in phytoestrogen can lead to onset of breast cancer. More the dose of alcohol more is the risk of cancer. Likewise, diets rich in fat leads to increased incidence of breast cancer as fats have cholesterol which is a precursor of estrogen and other steroids, thus fatty diet exposes breast to more and more estrogen causing breast cancer. Moreover, intake of dietary fibre is found to decrease breast cancer that's why incidence of breast cancer is less in Asia and Africa as their food is full of dietary fibre while in western countries dietary fibre intake is very less which leads to breast cancer.
- **Genetic factors:** It is found in the study that females with more and more first degree affected relatives are more prone to get affected by breast cancer.
- **Obesity, lifestyle and physical activity:** It is observed that sedentary life style, lack of exercise leads to obesity which further is responsible for onset of breast cancer because obesity means increase in fat tissue which has cholesterol. Cholesterol forms estrogen

which in excess causes breastcancer.

- **Endocrine endogenous factor:** Females who don't breast feed their babies and infertile females are more prone to get breast cancer. Late menarche and early menopause associated with first full term pregnancy at younger age are protective factors against breast cancer because it will reduce the exposure of breast with estrogen as it is reduced in pregnancy. Moreover, females having many kids will have very less chance of developing breast cancer due to same reason.
- **Endocrine exogenous factor:** Increased use of oral contraceptives before age of 20 years can be predisposing factors for onset of breast cancer, as oral contraceptives contain increased amount of estrogen which causes breast cancer. Hormone replacement therapy also increases breast cancer incidences.
- **Mammographic density:** After reproductive age it becomes risk factor for breast cancer. It is found that females with more than 75% breast density are found to be more vulnerable to breast cancer incidence in comparision to females with less breast density.
- **Benign breast disease:** Previous diseases of breast like fibroadenoma and fibrocystic disease are known to promote incidences of breast cancer. Fibrocystic disease along with epitheliosis and dysplasia creates pre-malignant conditions.
- **Steroid hormone and their receptor:** The role of steroid hormones in pathogenesis of breast cancer is well known. Fat cells contain aromatase enzyme which forms oestradiol from cholesterol. As ageing increases, increased content of fat cells in older post menopausal women, also increases oestradiol which further increases incidence of breast cancer.

1.1.3. WHO classification of BC- 5th edition (2019): (Muller et al, 2022)

- **Invasive BC of no special type (IBC-NST**) - It is a tumor of breast along with specific morphological features are considered in this type: rich in lipid, oncolytic, rich in glycogen, sebaceous, pleomorphic, choriocarcinomatouscharacteristics, cancer with melanocytic cells. There is no distinct subtype.
- **Invasive BC with medullary patterns**: Medullary cancer with distinct medullary features, atypical medullary cancer is categorized under this. All these show similar histologic features with basal molecular profiles and mostly with BRCA1 mutations.
- **Neuroendocrine Tumors**: Primary true neuroendocrine neoplasm inside breast is rarely found. They can be divided as properly differentiated cancer of neuroendocrine system (for

eg. Atypical carcinoid and carcinoid type) and less differentiated neuroendocrine tumors (foreg. large cell carcinoma and small cell carcinoma). Specific neuroendocrine features and marker expression is required for diagnosis since degree of neuroendocrine differentiation varies among different tumors.

- **Well differentiated liposarcoma in phyllodes tumor**: Malignant phyllodes tumor can be diagnosed on the presence of cancerous heterologous elements as one of the basis among others. MDM2 and CDK4 amplifications, characteristic feature of well differentiated liposarcoma is foud to be absent in adipocytic differentiation inside the stromal part of phyllode tumor (It is otherwise similar to well differentiated phyllode tumor).
- **Mucinous cystadenocarcinoma**: It is invasive and rare subtype, characterized by presence of papillae and intra and extra cellular mucin, rounded tumor border, lack of myoepithelium and spaces of cystlined with cancerous columnar epithelium.
- **Tall cell carcinoma having reverse polarity:** Rare, invasive subtype characterized by presence of solid tumor nests, inside fibrous stroma which has core line by columnar epithelium.

1.1.4. Anatomy of breast: (Pandya S. et al, 2011)

In a woman, breast is pear shaped gland that produces milk, attached on chest wall on both the sides of sternum with the help of ligaments. It rests on chest muscle, pectoralis major. Different types of tissue are involved in the formation of breast namely glandular tissue (produces milk), Adipose tissue (maintains breast size), and Connective tissue (holds glandular and adipose tissue). Different parts form breast anatomy as follows:

- **Lobes:** 15 to 20 lobes surround nipple.
- **Lobules:** It is a glandular tissue.
- **Milk ducts:** These ductsbring milk from lobules to nipples.
- **Nipple:** Present in middle of areola, contains 9 milk ducts and nerves.
- **Areola:** It is dark colored circular skin, contains montomer's gland which secrete lubricating oil to prevent chafing.
- **Blood vessels:** Circulates blood to breast and chest.
- **Lymph vessels:** It transports lymph which helps body fight infection.
- **Nerves:** Contains many nerves which makes it sensitive.

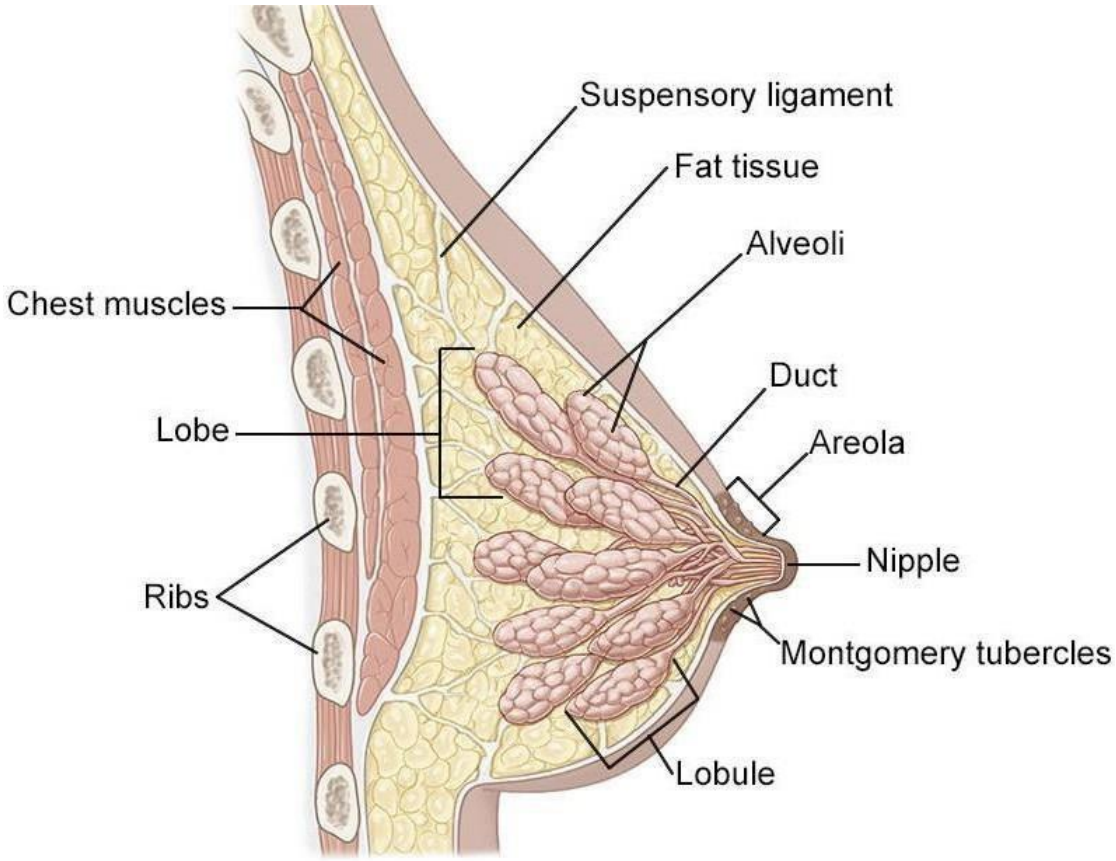

Figure 1.1: Structure of breast

1.1.5. Pathology of BC:

Breast cancer is mainly a disease of epithelial tissue categorized as carcinoma. It involves different types of lesions which differ in behavior and appearance. Breast in situ carcinoma can be either lobular or ductal. Distinction relies upon distinct cytologic lesion feature and growth pattern. Metastatic breast cancer has many histologic subtype likeductal (infiltrating), ductal, lobular (invasive), mucinous, papillary, tubular and medullary. Among these ductal (infiltrating) is most frequent type (Bleiweiss et al, 2013).

1.1.5.1. Different types of BC:

In situ ductal carcinoma (ISDC) includes proliferation of breast cancer cells inside the ductal system of breast with no invasion inside surrounding stroma. However, infiltrating ductal carcinoma invades into tissues, characterized by stellate and irregular shape. On the other hand, in situ lobular carcinoma and atypical lobular hyperplasia include cancer cell proliferation with probability of malignancy.

1.1.5.2. <u>Other histologic variants</u>: These include other histologic subtypes given as following:

- Tubular cancer
- Mucinous cancer
- Medullary cancer
- Tubulolobular cancer
- Micropapillary cancer
- Metaplastic cancer
- Adenoid cystic cancer
- Secretory cancer
- Apocrine cancer

1.1.5.3. <u>Pathophysiology of BC</u>: Normal cells of body aregenerally programmed to die at certain stage and time which is called as programmed cell death or apoptosis. Before apoptosis, cells are protected by certain protein complexes or pathways like PI3K/AKT pathway or RAS/MEK/ERK pathway, but sometimes genes responsible for these protein complexes get mutated and remain 'on' forever, thus cell remains alive and dividing always which leads to cancerous growth. In normal condition, PTEN protein deactivates PI3K/AKT pathway and apoptosis takes place but in cancer this does not happen (Lee A. et al, 2009). However, these mutations are found experimentally to be connected to exposure to estrogen. Overexpression of leptin leads to malignant growth in breast tissue (Jarde T. et al, 2011). Mutations in p53, PTEN and BRCA1/BRCA2 also known very well to cause breast cancer (Begg C.B. et al, 2008). GATA3 controls estrogen receptor expression in normalcondition but in malignant stage, differentiation is lost (Kouros-Mehr H et al, 2008).

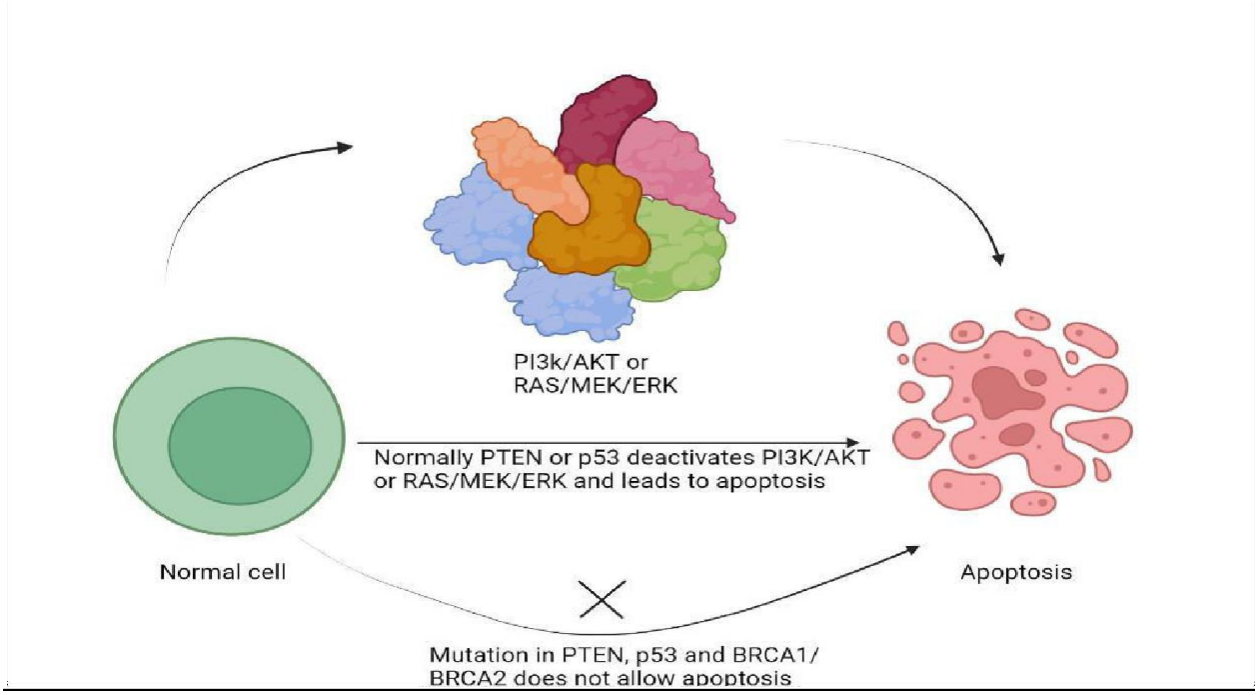

Figure1.2: Showing pathophysiology of BC

1.1.6. Risk factors associated with BC:

Risk factors can be of two types i.e. fixed risk factors (likesex and age) and modifiable risk factors (like alcohol intake) (Hayes J. et al, 2013).

- Being female and of older age is primary risk factor. Other important risk factors include lack of child bearing and breast feeding, genetics, higher hormone level, obesity and wrong dietary pattern and pollution (Reeder J.G. et al, 2008).
- **Lifestyle:** Lifestyle canbe improved by avoiding alcohol intake, tobacco smoking and taking diet rich in fats. Lack of physical activity leads to obesity which is a big cause of breast cancer. Eating healthy and balanced diet with good physical activity and avoiding alcohol and smoking is a key to reduce risk factors (Zhang Y.B. et al, 2020). Hormone therapy for menopause can also cause breast cancer. Radiation exposure also poses risk.
- **Medical condition:** Some breast conditions like lobular carcinoma in situ, atypical ductal hyperplasia and fibrocystic breast changes are benign conditions but can lead to breast cancer. Diabetes mellitus also poses risk (Anothaisintawee T. et al, 2013). Autoimmune disease like lupus erythematosus also causes increased risk (Bohm et al, 2011). Increased prolactin levels and estrogen levels also poses high chances of cancer of breast (Wang M. et al 2016) (William C. et al, 2013).

- **Genetics:** Genetics is cause of 5% to 10% cases. If the female has first degree relative as breast cancer patient in age of 40 or 50 then chances of getting breast cancer becomes double (Nelson et al, 2012). Female with BRCA1/BRCA2 mutations (Pasche et al, 2010) or p53 mutation, PTEN mutation, STK11 mutation etc. all these leads to breast cancer mostly (Gage M. et al, 2012).

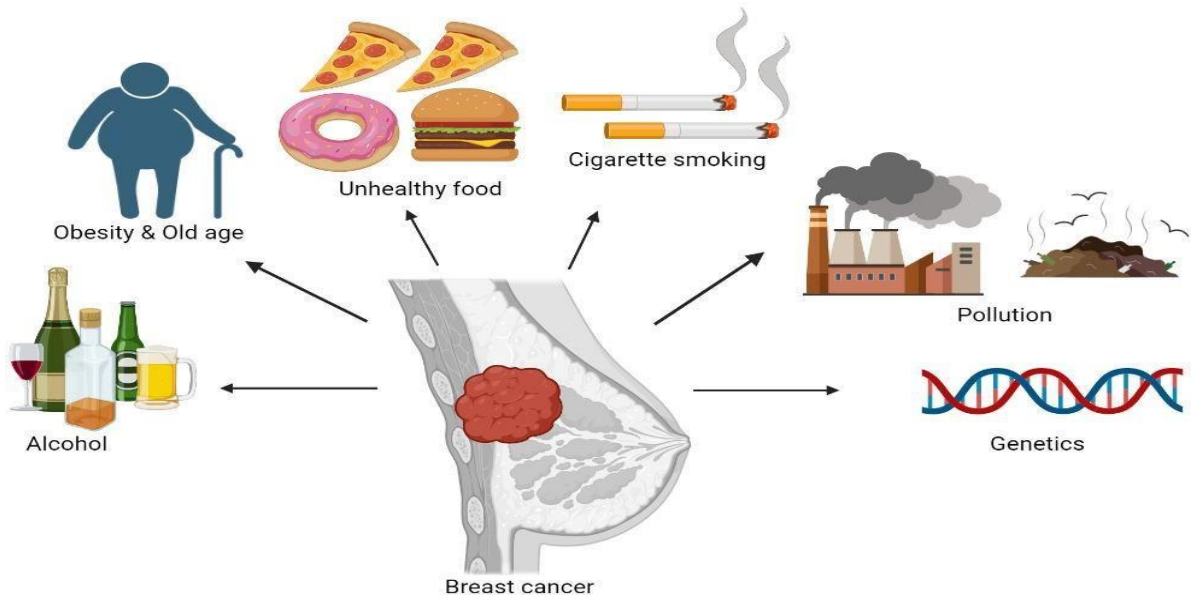

Figure 1.3: Risk factors of breast cancer

1.1.7. Clinical features associated with BC :

- Commonest symptomof cancer of breast is presence of knots in breast which must be detected by fingertips. Other symptoms include thickening of tissues, one breast lower or larger, changed shape or position of nipple, inverted nipple, skin dimpling around nipple, watery or bloody discharge from nipple, pain or swelling in or around armpit (Watson M. et al, 2008).
- In Paget's disease of breast, skin resembles like eczema, discoloration, redness, flaking of nipple skin takes place. In advance stage, itching, tingling, burning, increased sensitivity, pain, discharge and lump too (Kollmorgen et al, 1998).
- In inflammatory breast cancer, blockage of lymph vessels by cancer cells is found so, redness takes place in breast tissue although lump is not formed (Kleer C. G. et al, 2000).
- In Mammary secretory carcinoma (MSC), occurs exclusively in breast. It accounts for 80% of childhood breast cancer. MSC lesions are mainly painless, slow growing, invades nearby tissue (Knaus M.E. et al, 2021).

- In phyllodes tumor, hard, movable, cancerous lump in stroma of breast, based on appearance in microscope it can be benign or malignant (Lacroix M. et al, 2006).

1.1.8. Diagnosis of BC: (Saslow D. et al, 2004) (Yu Y. H. et al, 2010)

Early diagnosis of breast cancer is very crucial for treatment. It can be done in following ways:

- **Physical examination:** Doctor will see and touch the breast for any kind of lump or cyst which may or may not be cancerous. If there is pain in that it is tested further.
- **Microscopicanalysis:** Inmicroscopic analysis of breast, FNAC (fine needle aspiration and cytology) is done, in this needle is inserted into lump to aspirate fluid and examined under the microscope to know proper condition of the tissues.
- **Biopsy:** Tissues are taken out from the affected area of the breast and examined using core biopsy, in this a part of lump is taken out and examined. However, excision biopsy also can be done in which whole lump is taken out an examined.
- **Mammography:** In this X-rays are used to figure out the microcalcification of breast or to detect breast cancer.
- **Magnetic resonance imaging (MRI):** when other techniques of detecting breast cancer are inconclusive then MRI is done to conclude the diagnosis and final confirmation is done.

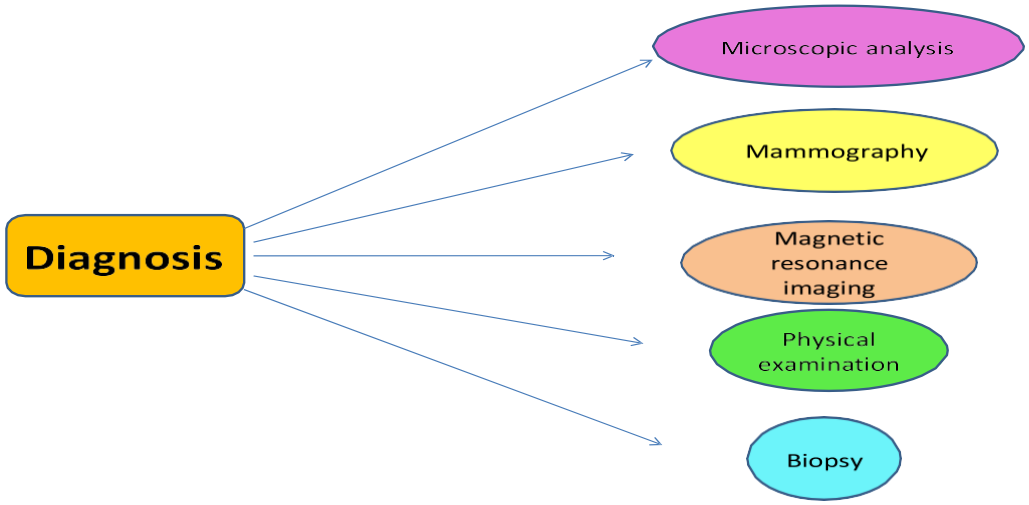

Figure 1.4: Diagnostic methods in breast cancer

1.1.9. Prevention strategies for BC:

- **Lifestyle:** Breast cancer can be prevented in women by maintaining control on body weight, avoidingalcohol use, having active lifestyle, breast feeding, enhanced physical activity (Eliassen A.H. et al, 2010), having diet rich in fruits, legumes, grains (Runowicz et al 2016), soy based food (Wu A. H. et al, 2008) and omega-3 polyunsaturated fatty acids are considered valuable measures for reducing breast cancer occurrence (Zheng et al, 2013).
- **Medication:** Few estrogen receptor modulators like tamoxifen is considered to decrease the risk of breast cancer, but they have risk of thromboembolism and endometrial cancer thus not recommended in low risk women (Nelson et al 2013). Although, aromatase inhibitors are considered better to prevent breast cancer as they lack such side effects (Mocellin S. et al, 2019).
- **Pre-emptive surgery:** In this precautionary measure both breast will be removed before any kind of diagnosis of breast cancer in women having BRCA1 and BRCA2 mutations as peoplehaving these mutations are highly prone to get breast cancer during their lifetime. So, BRCA testing is very much recommended in women having family history in genetic counseling (Meijers-Heijboer et al, 2022).

1.1.10. Treatment of BC: (Moo T.A. et al, 2018)

Treatment of breast cancer is multidisciplinary. After diagnosis when the stage of breast cancer is confirmed thenit is planned that which kind of treatment should be given to patient. Stage 4 is considered incurable. For other stages of breast cancer, extent of metastasis is observed for proper treatment. In stage 1 and stage 2 patients, tumor resection is primarily done, with optional mastectomy. Approaches used for treatment are as follows:

- **Local therapy:** In case of invasive breast cancer, both breast conservation therapy (BCT) and mastectomy are the options depending on the stage. Locoregional recurrence (LRR) isobserved in case of BCT more than mastectomy, but mostly it depends on presence of hormone receptor (HR). In HR-ve and HER2-ve cancers i.e. triple negative breast cancers (TNBC), LRR is highest in comparision to HR+ve and HER2-ve types. Hence, BCT can be done for majority of patients.
- **Breast conservation therapy:** In BCT tumor is excised, followed by whole breast irradiation (WBI). In this tumor is cut with cosmetic outcomeso that it can be irradiated after tumor removal and should be able for follow up to detect tumor recurrence. Although there are some contraindications for BCT like BRCA1/2 mutation, active scleroderma etc.

- **Adjuvant radiation in BCT:** Along with BCT, adjuvant radiation should be given for more assurance to get rid of tumor cells but some conditions are contraindications in its usage likepresence of connective tissue disorders, pregnancy and prior chest wall irradiation.
- **Mastectomy:** It is done mainly in case of invasive carcinoma. There are different options for people having mastectomy like complete mastectomy, nipple-areolar and skin sparing mastectomy.
- **Post mastectomy radiation (PMR):** After mastectomy breast tissues are irradiated to assure complete removal of cancer cells in case of both early and advanced cancer. Patients with large tumor size and with axillary lymph nodes are more likely for LRR. Thus post mastectomy irradiation is highly recommended.
- **Axilla staging and management:** As initial site of metastasis is axillary nodes so proper management is required. Biopsy ofsentinel lymph node is done for staging of axilla. It should be negative for assurance of absence of LRR.
- **Neoadjuvant chemotherapy (NAC):** In this surgery is combined with chemotherapy. Main aim is to convert inoperable tumors operable and to decrease the incidence of LRR in patients. BCT can be done if NAC is done at early stage examined by mammography or MRI. NAC decreases axillary metastasis significantly. These systemic therapies depend on disease biology and disease burden.
- **Chemotherapy:** Systemic chemotherapy is mostly recommended in high risk patients. Standard drugs containing taxanes and anthracyclines are chemotherapeutic options for patients. Adjuvant chemotherapy is proven to reduce mortality and LRR in breast cancer patients. Node positive patients of breast cancer mostly used to get chemotherapy than negative node patients due to poor prognosis.
- **Biologic and targeted therapies:** HER2 targeted therapy is given to HER2 positive patiets with chemotherapy. It changed the prognosis completely**.** Monocloal antibodies also used with targeted receptor therapy for better results.
- **Endocrine therapy:** Endocrine therapy is mostly recommended in patients who have hormone receptor. Tamoxifen is used as an adjuvant to reduce the occurrence of LRR. Longer duration of endocrine therapy gives more benefits. It stops the growth of cancer cells which use hormones to grow.

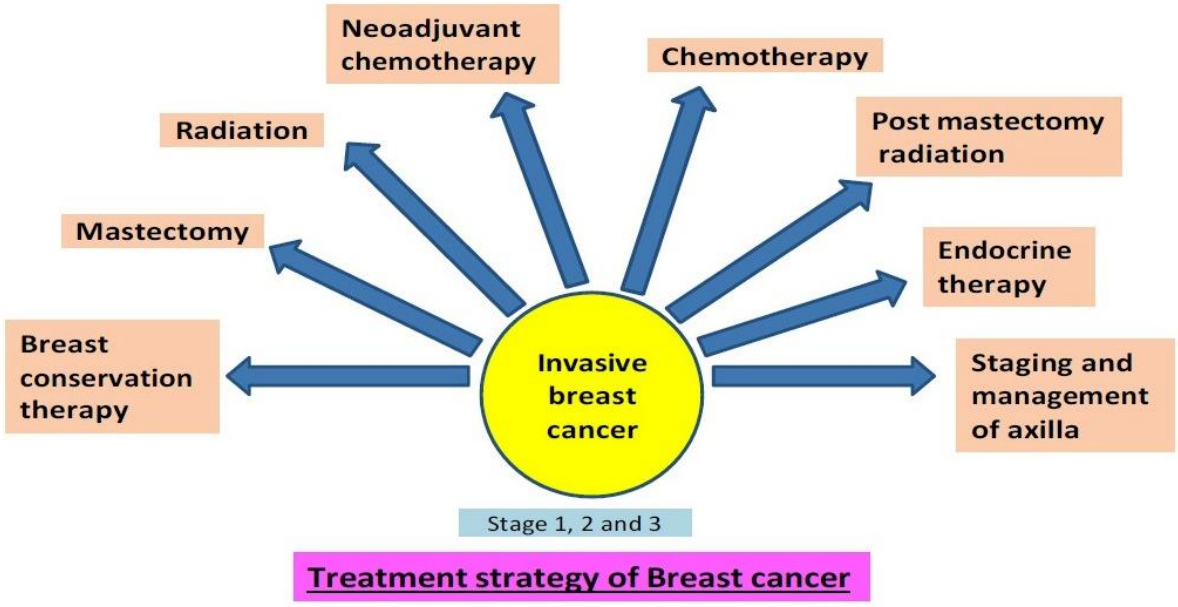

Figure 1.5: showing different ways of treating breast cancer

1.2. Triple negative breast cancer (TNBC):

The characteristic feature of TNBC is absence of estrogen and progesterone hormone and HER2/neu receptor. As all three receptors are found absent that is why this type of cancer is called as triple negative breast cancer. TNBC has completely different pathological and clinical features and is considered as clinical problem due to its aggressive behavior, poor prognosis and absence of targeted therapies. It falls under basal like subtype of breast cancer but not synonymously. It is mostly associated with tumors having BRCA1 mutation in young ladies of America and Africa. This clue can be used for treatment and prevention (Irwin et al, 2008). Metastasis to brain and viscera is more common than normal breast cancer. Taxanes and anthracyclines are used as major drugs for chemotherapeutic treatment. Several inhibitors like Poly ADP ribose polymerase inhibitor, c-kit inhibitor. Epidermal growth factor inhibitor, mTor inhibitor, Raf/Mek/Map kinase inhibitors etc. are used as therapeutic option for TNBC (Chacon R.D. et al, 2010). 15-20% of different breast cancers are TNBC (Sporikova Z. et al, 2018). TNBC forms hetrerogeneous type of breast cancer, very difficult to treat (Garrido-castro et al, 2019). Hormone therapy, which is used for breast cancer, isnot useful for TNBC. In initial stage, surgery, chemotherapy and radiotherapy, all are used as treatment options but in later stages when cancer becomes malignant then chemotherapy remains only option.

TNBC has unique relapse pattern in which replapse is much high in 3-5 years, butlessens

further after 5 years in comparision to breast cancer (Hudis CA et al, 2011).

1.2.1. Classification of TNBC:

TNBC features resemble basal subtype in being receptor negative and also produce basal cytokeratins (Ovcaricek T. et al, 2011). 85% of basal tumors are found to be TNBC. Thus, classification of TNBC subtypes depending upon basal like tumors is as follows (Ensenyat-Mendez M. et al, 2021):

- Luminal androgen receptor
- Mesenchymal
- Basal like 1
- Basal like 2

However, most of the TNBC is invasive breast cancer of no special type. Following types of breast cancers have probability of being TNBC (Plasilova ML et al, 2016).

- Inflammatory
- Apocrie adenocarcinoma
- Medullary carcinoma
- Metaplastic
- Adenoid cystic carcinoma

1.2.2. Risk factors of TNBC:

- All age groups are similarly affected by TNBC. Basal type and BRCA mutations related TNBC is present in young women whereas normal like having high apocrine level i.e. neuroendocrine TNBC is found in older women (Hudis CA et al, 2011).
- In a US study, it was observed that among young ladies, American, African and Hispanic women have high risk of getting TNBC. Moreover, American African tends to have poorer prognosis than other races (Chustecka et al, 2008).
- Another very well established risk factor of TNBC is germline mutations. These arechanges within the genes which get transferred into the offspring. As BRCA1/BRCA2 mutations are present in different types of cancers like breast, pancreas, and ovary and prostate. So, it is consideredas high risk for TNBC (Pruss D et al, 2014).
- Mutations in MDM4 and 19p13.1 loci are also found linked with TNBC only instead of other types of breast cancer. Thus unique germline mutations are associated with TNBC

(StevensKN et al, 2013).

- Use of oral contraceptives for more than one year by women of less than 40 years are more prone to get TNBC than women 41-45 years of age taking oral contraceptive for less than one year (Dolle JM et al,2009).

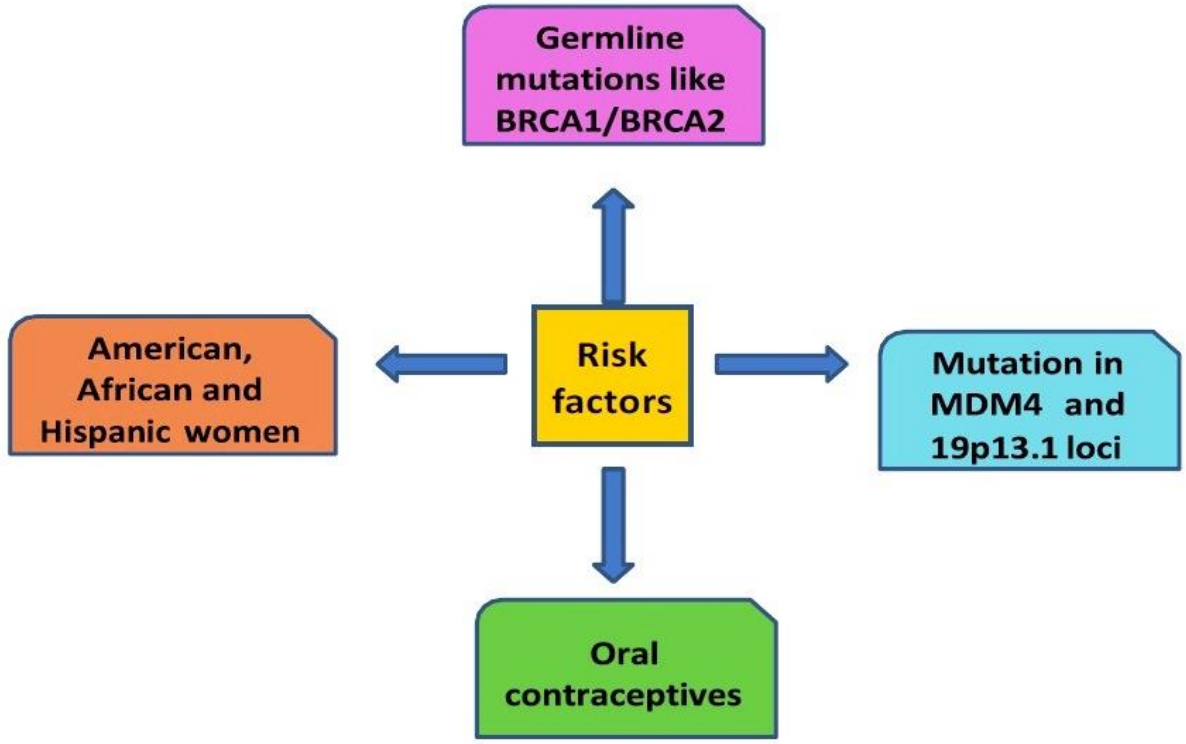

Figure 1.6: Risk factors of TNBC

1.2.3. Prognosis of TNBC: (O'Reilly D. et al, 2021)

TNBC is a highly metastatic cancer type with very poor prognosis. Its recurrence rate is very high during first five years after treatment than other types of breast cancer, but after five years recurrence rate is much less than other types of breast cancer. During first three years recurrence rate is highest afterwards it decreases. Survival of TNBC patients is a 5-year program. Rate of survival of TNBC patientsis compared with the healthy subjects (without breast cancer). It is based on the stage of diagnosis. If cancer comes back, then this below statistic will not be applied.

Table 1.1: Showing survival rate:

Stage of diagnosis in patient	5 year survival of patient
LocalizedTNBC	91% chances
RegionalTNBC	65% chances
DistantTNBC	12% chances
All stages of TNBC	77% chances

25% of cases of localized cancer may relapse into distant metastasis which occurs in stage IV cancer. medium survival is 12 months. Metastasis in TNBC usually takes place in lung, liver, brain etc, instead of bone, a distinct metastatic feature of TNBC unusual for other breast cancer types.

1.2.4. Treatment of TNBC:

Treatment of TNBC depends on the stage of cancer in patient as follows:

- **Early stage TNBC:** Gold standard for treatment of TNBC in early stage is surgery along with radiotherapy and chemotherapy. Surgery is mainly done either by lumpectomy or mastectomy. Overall survival was found to be higher for lumpectomy with adjuvant therapy, than mastectomy with adjuvant therapy (Guo L. et al, 2021). Neoadjuvant chemotherapy, a platinum based regimen is mostly used as a breast conserving therapy. However, betterment of breast conservation therapy is based upon responses from patients. Cancer cells of TNBC can be completely abolished in early stage by chemotherapy, however it does not always reflect into improvement in overall survival (Bergin A. et al, 2019).
- **Late stage TNBC:** Late stage TNBC becomes metastatic. Treatment schedule is designeddepending upon PD-L1 (Programmed death cell ligand 1) protein is present in TNBC or BRCA gene mutation. Immunotherapy is used to kill cancer cells. Immune checkpoints inhibitors and chemotherapy is used to treat advanced metastatic TNBC. PARP inhibitors are also used as better option than chemotherapy for advanced TNBC (Moy B. et al, 2021).

  Metastatic TNBC + PD-L1: Immune checkpoint inhibitor + chemotherapy **Metastatic TNBC + PD-L1:** Chemotherapy + sacituzumabgovitecan **Metastatic TNBC + BRCA:** Chemotherapy + PARP inhibitor

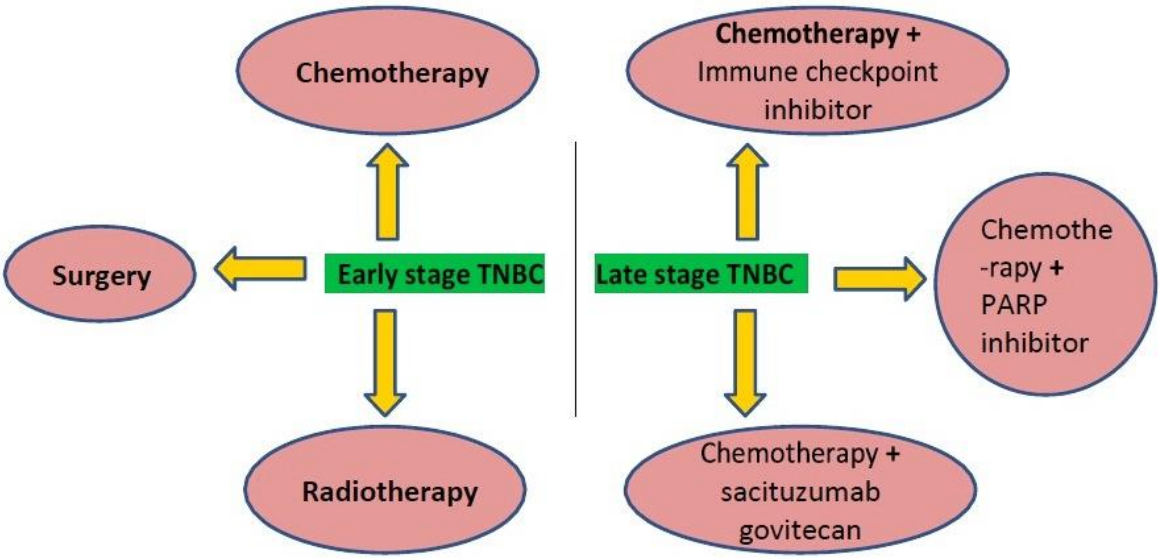

Figure 1.7: Treatment of TNBC

1.3. Different Protein Receptors for BC and their clinical implications:

Receptors are proteins which are present on normal breast cells and some breast cancer cells too, for binding with the corresponding hormone for growth of the cells. There can be various kinds of receptor like estrogen receptor (ER), progesterone receptor (PR), HER2 neu receptor (human epidermal growth factor receptor 2 neu), vitamin D receptor etc. These receptors are most prevalent in breast cancer tissues and thus are mostly measured. These receptors are used for the treatment purpose as hormone therapy drugs can be used for decreasing estrogen or progesterone hormone content which ultimately acts to decrease breast cancer cell growth and to improve prognostic features. This type of treatment is possible only for breast cancerpositive for hormone receptor. However, in case of cancersnegative for hormone receptor this treatment cannot be done. If the cancer is ER+ then it can get growth signal from estrogen, however if cancer id PR+ it can get growth signal from progesterone. So, hormonal therapy is based on the inhibitors of these hormones thatmean inhibitors will be used as a drug to block the binding of the hormone onto the receptor, so that receptor will never get signal for growth and cancer will stop proliferating (Stanford et al, 1996). Patients with estrogen receptor positive cancer in initial years after diagnosis usually has lesser recurrence rate, however in later years recurrence

is increased so overall survival is balanced (Osborne et al, 1998). However, breast cancers with vitamin D receptor are ligand independent. Vitamin D controls growth of normal breast cell and can stop growth of breast cancer cell also. Women who have low levels of vitamin D have greater chances of getting breast cancer. Vitamin D especially vitamin D3 can lessen the risk of breast cancer (Buras R.R. et al, 1994). HER2+ breast cancer contains receptor HER2 that promotes growth of breast cancer cells. ER and HER2 are the most prominent driver of cancer growth thatswhy suitable for hormone targeted therapy. Prognosisof HER2+ breast cancer patients is excellent as 90% of them remains disease free in early stage. HER2+ cancer spread faster than HER2-, but responds well for treatment (Mitri Z. et al, 2012).

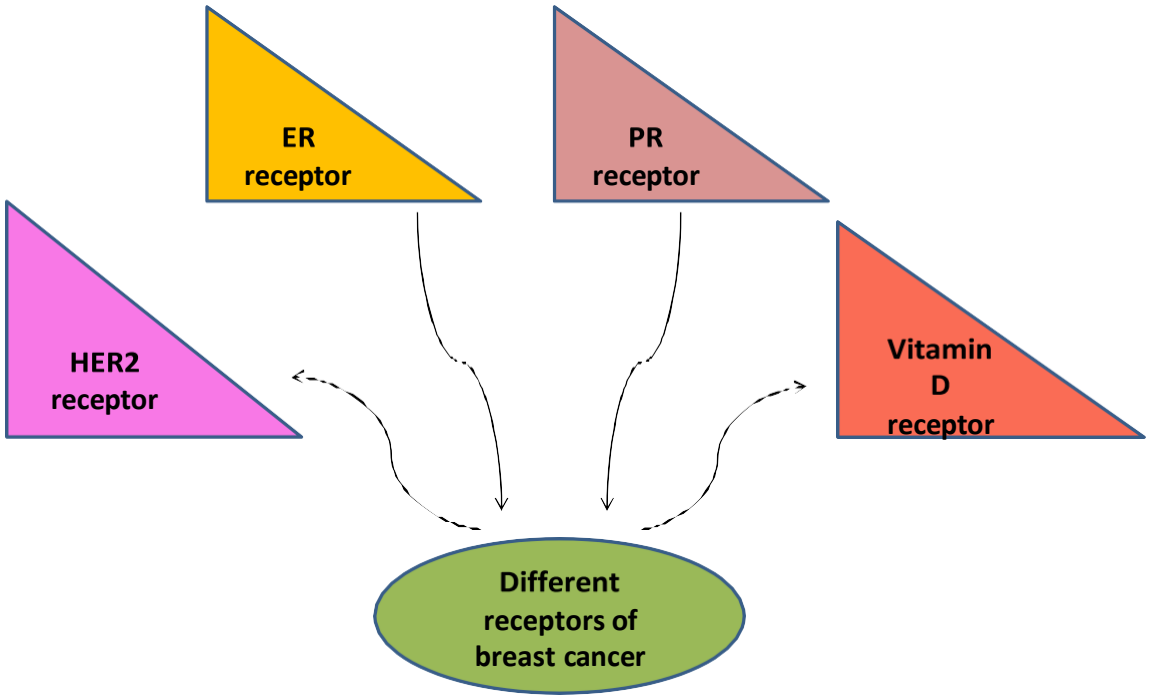

Figure 1.8: Different receptors of BC

1.3.1 : Clinical implications of receptors of BC:

Expression of hormone receptor in different tumors allows non cytotoxic treatments targeting tumor biology. Hormone receptor profiling helps in therapy and avoid overtreatment. Combination therapies which helps in overcoming resistance can also be developed by understanding the crosstalk between receptors. In few cancer, hormone modulation acts as effective fertility sparing option in young patients. Presence or absence of hormone receptors determines the subtype and related treatment plan of breast cancer. ER+/PR+ cancers like breast cancer, ovarian cancer and endometrial cancer are mostly slow growing and contains better prognosis. These cancers are usually treated with hormone therapy such as tamoxifen and aromatase inhibitors. Furthermore, HER2+ cancers are aggressive and usually treated with HER2 targeted therapy like trastuzumab, pertuzumab etc. However, in TNBC these three receptors are absent and thus it is treated by chemotherapy instead of hormonal or targeted therapy. Vitamin D receptor (VDR) is usually expressed on normal breast tissue and cancerous breast tissue. Higher VDR expression results in better prognosis i.e. slow growth and longer survival.

1.3.2: Major hormonal receptors for other types of Cancer and its implications in therapy:

Hormone receptors are crucial in the progress and treatment of several types of cancer, other than breast cancer also. So, proper understanding of expression and role of these receptors can augment effective therapy.

1. Estrogen and Progesterone Receptors (ER/PR)

Several endometrial tumors and prostate cancer show ER and PR. Progestins are used as effective hormonal therapy, mainly in low grade and early stage tumors. Few low grade serous ovarian cancer also express ER and PR. In this, antiestrogen therapy like tamoxifen and aromatase inhibitors are used as therapeutic option (Bonkhoff, H. et al, 2018, Liao, W. et al, 2023). Progesterone hormone found involved in causing ovarian cancer (Mauro, L. J. et al, 2023).

2. Androgen Receptor (AR)

AR is centrally involved in the growth and development of prostate cancer. Androgen deprivation therapy (ADT) includes LHRH agonists and antiandrogens like enzalutamide is the base of treatment. Few TNBC tumors called LAR subtype express AR. AR inhibitors like bicalutamide or enzalutamide are getting explored in trials as therapeutic option. Salivary duct

carcinoma is generally AR positive. Antiandrogen therapy is usually given in recurrent/metastatic disease. There is increasing evidence that thyroid hormones work with androgen receptors to regulate gonadal differentiation and reproductive function. Studies show that thyroid hormone interact with the AR promoter region and increase AR expression. Crosstalk between thyroid hormone and androgen receptor promote development of prostate cancer (Torabinejad, S. et al, 2023).

3. Thyroid Hormone Receptors (THRs)

In thyroid cancer, thyroid hormone receptors are critically involved in modulating differentiation and proliferation. In papillary and follicular thyroid cancer, TSH is involved. Treatment with levothyroxine suppresses TSH which reduces recurrence risk in differentiated thyroid cancer (Mousa, S. A. et al, 2021). Thyroid hormone receptors are also found associated with the development of lung cancer (Deligiorgi, M. V. et al, 2021)

4. Glucocorticoid Receptor (GR)

In hematologic malignancies like acute lymphoblastic leukaemia, glucocorticoids cause apoptosis in lymphoid cells. For this, steroids like prednisone and dexamethasone are gold standard for chemotherapy regimen. In solid tumors also, GR signaling may contribute to resistance to chemotherapy and targeted therapies. Glucocorticoid receptors are considered as potential target for combination therapies to overcome resistance (Olivas-Aguirre, M. et al, 2021). GR regulates and interacts with LEDGF/p75 to promote docetaxel resistance in prostate cancer (Sanchez- Hernandez et al, 2023).

5. Luteinizing Hormone-Releasing Hormone (LHRH) Receptors

In prostate cancer, LHRH receptors and its gene expression were found to be of high incidence. Agonists/antagonists are used to suppress production of sex hormone. LHRH receptors are common in hormone sensitive prostate and premenopausal breast cancer treatment (Halmos et al, 2000). In ovarian and endometrial cancers, expression of LHRH receptors may allow direct targeting with LHRH analog-drug conjugates and agonists (Emons, G. et al, 1990).

6. Somatostatin receptor (SS-R)

Somatostatin receptors (SS-R) were observed in tissue sections or membrane homogenates in many hundred differen tumors. SS-R were found mostly in neuroendocrine tumors, like TSH and GH releasing pituitary tumors, endocrine gastroenteropancreatic (GEP) tumors, pheochromocytomas, paragangliomas, small cell lung carcinomas and medullary thyroid

carcinomas (MTC). SS-R were also expressed in a majority of malignant lymphomas, in breast tumors and in several brain tumors (all meningiomas, most astrocytomas). SS-R mediates roles in human tumors according to the type of tumor. SS-R in GEP tumor and pituitary tumor inhibits hormone secretion with antiproliferative effects. In meningiomas, SS-R upon activation inhibits forskolin-stimulated adenylate cyclase activity and also stimulates proliferation weakly. Whereas, in animal models and cell lines of lung tumors, lymphomas and breast cancer, SS-R mediates antiproliferative effects. Clinical implications of SS-R in tumors are manifold: (1) It acts as a predictive marker for efficient therapy with octreotide in GEP and pituitary tumors (2) It also acts as a diagnostic marker for pathobiochemical classification of tumors *in vitro*, and *in vivo* using scanning techniques (3) acts as a prognostic marker and (4) also acts as a potential radiotherapeutic target (Reubi, J. C. et al 1992).

7. Insulin-like growth factor (IGF) binding proteins (IGFBPs)

Insulin-like growth factor (IGF) binding proteins (IGFBPs) are a family of six proteins which function as transport proteins for IGF-I and IGF-II in the circulation and also regulate their access to IGF-I, the potentially oncogenic receptor. 'Free' (unbound) IGFs are short lived, whereas IGFBP-bound IGFs are much more stable. IGFBPs changes function of cell by both IGF-dependent mechanisms, which modulate IGF1R signalling, and IGF-independent mechanisms, which does not result in any change in IGF1R signalling. Several studies have shown that IGFBPs or their mRNAs can be useful as prognostic markers. No cancer is reported with IGFBP gene mutation, but epigenetic silencing of intracellularly both. They inhibit cancer progression by inducing apoptosis and stopping cell proliferation, however, in other cases they promote cell survival and stimulate cell proliferation. IGFBP act as attractive drug target (Baxter, R. C., 2014).

8. Protein Kinases (PK)

Protein kinases are enzymes which phosphorylate different proteins and regulate several cellular processes like cell proliferartion, division, survival, metabolism and apoptosis. Tyrosine and serine- threonine protein kinases perform this phosphorylation in cells. Human protein kinases constitute complicated system with internal and external interactions. Alterations in funntion of this enzyme result in several pathological changes and causes disease. It is shown to be involved in several diseases including cancer (Shchemelinin, I. et al, 2006). Drugs which target tyrosine kinase can act as potent anticancer drugs. Inhibitors of PI3K, MEK, and ERK signalling pathways are under clinical trials. Thus, drugs targeting protein kinases act as a promising area of cancer therapy (Singh, V. et al, 2017).

9. G protein-coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) are involved in many biological and pathological processes. They are one of the most desirable drug target. Recently, it was found that many GPCRs like chemokine receptors, endothelin receptors and lysophosphatidic acid receptors were involved in tumorigenesis and metastasis of different human cancers like lung, colon, gastric, thyroid, pancreatic, prostate, melanoma etc. GPCRs modulate different processes like proliferative signaling, evasion of growth suppressors, replicative immortality, initiation of angiogenesis, resistance to apoptosis and activation of invasion and metastasis which are cosidered as hallmarks of cancer. Experiments have shown that GPCRs have role in the regulation of the differentiation, maintenance and pluripotency of stem cells of cancer. Current drugs targeting GPCRs have shown excellent therapeutic benefits as GPCRs, like many other kinds of cell surface proteins, can be targetable in several malignancies (Chaudhary, P. K, et al, 2021).

10. P2 receptor

P2 receptors are composed of ligand-gated ion channel type (P2X receptor) and G protein-coupled metabolite type (P2Y receptor). Both these receptors have played important roles in the prostate cancer and colorectal cancer microenvironment in recent years. P2X and P2Y receptors can contribute to prostate cancer's growth and invasiveness and antitumor response. P2 receptors are activated by extracellular ATP, are involved in cancer development, progression and metastasis (Wang, Z et al, 2023).

11. **Toll like Receptors** (TLR)

Toll-like receptors (TLR) are proteins of innate immunity, known as pattern recognition receptors. They utilize specific patterns present on pathogens called as pathogen- associated molecular pattern and release certain chemicals which causes inflammation. Toll-like receptors removes infected cell from body and thus stop spreading infection. Certain polymorphisms in toll-like receptor makes cell more sensitive to develop oral cancer. The study has revealed that toll-like receptors like TLR5 and TLR7 were involved in suppressing oral cancer while toll-like receptors like TLR2 and TLR4 were involved in progression of oral cancer. Thus, Toll-like receptors can act as potent target molecules in drug designing for oral cancer (Bhardwaj, A. et al, 2024). TLR also acted as potent prognostic biomarker for

gastric cancer (Eskuri, M. et al, 2024, Castaño-Rodríguez, N, et al 2014).

1.4. PPAR-γ receptor

1.4.1. Introduction of PPAR-γ receptor

PPAR (Peroxisome Proliferated Activated Receptor) gamma is a protein in type IInuclear hormone receptor subfamily, also known as the glitazone reverse insulin resistance receptor (Michaelik L. et al, 2006). It functions as a transcription factor activated by ligand, when it heterodimerizes with retinoid x receptor (RXR) and binds on PPRE sequences on DNA, a process essential for all PPAR-γ-DNA interactions (Yang W. et al, 2000). Itswell established function is to modulate insulin sensitivity and in mediating adipocyte differentiation (Mandrup S. et al, 1997). It is encoded by PPARG gene. PPARG gene has two isoforms PPARG1 (expressed in all tissues except muscles) and PPARG2 (mainly found in adipose and intestine). Overall, PPARG gene is expressed in colon, adipose tissue and macrophages (Fajas L. et al, 1997). PPAR-γ regulates glucose and lipid metabolism and maintains homeostasis (Gampe Jr. et al, 2000), and is also involved in adipocyte differentiation (Tontonoz P. et al, 2008). PPAR-γ is well researched target for antidiabetic drugs like thiazolidinediones for type II diabetes. It is also found potent for treating chronic inflammatory and metabolic diseases like inflammatory bowel disease and metabolic syndrome respectively, due to its involvement in processing of these diseases. Malfunction of PPAR-γ related regulatory functions are involved in development of atherosclerosis as well as different cancers such as breast, colorectal and prostate cancer (Han L.et al, 2017, Decara J. et al, 2020).

1.4.2. Structure of PPAR-γ receptor

PPAR-γ contains activation domain (A/B domain and AF-1 region) which is independent of ligand, a ligand binding domain (LBD)(E/F-domain and AF-2 region) which depends on ligand, a DNA binding domain (DBD)(C-domain) and a hinge region (D domain) (Lewis SN et al, 2010).Array of ligands can bind in large binding pockets in PPAR-γ. Conformational change triggers in PPAR-γ on ligand binding, especially in activation function 2 (AF 2) domain, which helps in increased involvement of co-regulatory factors for regulation of transcription of genes (Kroker AJ et al, 2015). Two isoforms of PPAR-γ, i.e. PPAR-γ 1 and PPAR-γ 2, differs from each other by the presence of extra 30 amino acids at N terminal of PPAR-γ 2, which makes PPAR-γ 2 more potent and capable of increased adipose selectivity, due to which it acts as key player for adipocyte differentiation (Tontonoz P. et al, 2008). LBD of PPAR-γ has 13 alpha helices and 4 short beta strands (Gampe etal, 2000). It contains a

binding pocket of T-shaped with volume of ~1440 Å3 (Itoh T. et al, 2008), which is bigger than many other nuclear receptors (Pochetti G. et al, 2007) and allows PPAR-γ to interact with many ligands (Murphy GJ et al, 2000). PPAR-γ LBD domain contains helical sandwichshape, present on C terminal end of PPAR-γ, made of 250 amino acids (Lewis et al, 2010).

1.4.3. Features of PPAR-γ

- **Ligand activity**

PPAR-γ ligands can be of three types: full agonist, partial agonist and antagonist (Lewis et al, 2010). Full agonists bind the best way on PPAR-γ, thus activates it with high efficacy and strength (Pochetti G. et al, 2007, Gampe Jr. et al, 2000). Full agonists make hydrogen bonds withH323, S289, H449and Y473 residues of the PPARγ-LBDand uses polar functional groups of ligand itself (Pochetti G. et al, 2007), and causes very dramatic conformational difference (Gampe Jr. et al, 2000), whereas, partial agonistscauses subtle change in conformation (Gampe Jr. et al, 2000), low potency as well as efficacy (Pochetti G. et al, 2007). On the other hand, antagonists do not activate PPAR-γ, thus causes either no conformational change or very slight to accommodate co-repressors (Lewis et al, 2010, Xu HE et al, 2002). However natural ligands of PPAR-γ include prostaglandins, eicosanoids and fatty acids etc (Lewis et al, 2010, Itoh T. et al, 2008, Pochetti G. et al, 2007, Murphy GJ et al, 2000, Xu HE et al, 2002). Regulation of PPAR-γ ligand binding is done between the A/B domain (which is present at N- terminal), situated close to DBD, as well as the LBD present at carboxyl-terminal (Shao D. et al, 1998).

- **Co-activators/Co-repressors**

Hydrophobic residues present at H3, H3', H4, and H12 helices create a groove called as the coactivator site in PPAR-γ. When ligand binds, stabilization of AF-2 domain is achieved, this is required for coactivator interaction (Gampe Jr. et al, 2000). When agonist binds then it assists binding of coactivators and chromatin remodeling factors also to start transcription (McKenna NJ et al, 2002). Genes are silenced by PPAR-γ by using corepressors, and then AF-2 region is stabilized by binding of antagonist, thus prevention binding of coactivator and transcription activation (Xu HE et al, 2002). General coactivators ofPPARgamma are TRAP220, CBP/p300 and the SRC family whereas general corepressors involve NCoR, SMART and RIP140 (Tontonoz P. et al, 2008).

- **Function of PPAR-γ**

PPAR-γ is known as a major regulator of glucose and lipid homeostasis (Gampe Jr. et al, 2000).

It controls gene expression of genes involved in fatty acid metabolism, which includes lipoprotein lipase (LPL), fatty acid binding protein (aP2) of adipocyte and acyl-CoA oxidase. PPAR-γ deletions inspecific tissues results in resistance for insulin, lipodystrophy and less life of mature adipocytes (Tontonoz P. et al, 2008, Desvergne B. et al, 1999, Lewis et al, 2010). It also alters insulin sensitivity, cell proliferationand inflammatory processes and mediates adipocyte differentiation (Shao D. et al, 1998). Macrophage activation and inflammatory cytokine production is inhibited by ligands of PPAR- γ (Murphy GJ et al, 2000).

1.4.4. Involvement of PPAR-γ receptor in different diseases

PPAR-γ is found involved in different disease conditionslike hyperglycemia, hyperlipidemia (Lehrke M. et al, 2005, Kim JH et al, 2015), inflammatory diseases of bowel (Su CG et al, 1999), colon (Dubuquoy L. et al, 2006) etc, and in overcoming neurological (Krishna S. et al, 2021) and biochemical deficits (Ding Y. et al, 2020) and cancer (Chi T. et al, 2021). Firstly, identified PPAR-γ ligands are synthetic thiazolidinediones (TZD). The most potent among all TZDs was rosiglitazone.

Type II diabetes, characterized by increased leves of triglycerides and free fatty acids in blood, can be treated by TZDs to reduce the same characteristics and help in increasing insulin sensitivity (Murphy GJ et al, 2000). TZDs also found to decrease pro-inflammatory cytokines and increase levels of GLUT- 4 (Lewis SN et al, 2010). Levels of PPAR-γ are high in epithelium of colon. In colon, PPAR-γ is concerned with regulation of inflammation and immune response. Presence of inflammatory cytokines in colon is the reason of inflammatory bowel disease. Expression of PPAR-γ found defected in diseases like ulcerative colitis, which is also mandatory for aminosalicylate activity incase of inflammatory bowel disease. Colon inflammation can be controlled by TZDs (Su CG et al, 1999). Psoriasis and colitis treatment can be done by agonists of PPAR-γ which inhibits inflammation and decreases cytokine production. PPAR-γ stops action of nuclear factor NFκB, which is found in increased concentration in patients of ulcerative colitis (Sartor et al, 2006). PPAR-γ can be used for treating other differentdiseasesrelated to inflammation. PPAR-γ agonists can modulate the immune response. Rosiglitazone alongwith adiponectin treats renal diseases, autoantibodiesproduction and atherosclerosis. Allthese are characteristic feature of Systemic Lupus Erythematosus (SLE), an inflammatory autoimmune disease (Aprahamian T. et al, 2009). PPAR-γ ligandsare considered as potential measure for cancer treatment due to several reasons, like their ability to inhibit angiogenesis, and decreasing proliferation and metastasis of

solid tumors (Murphy GJ et al, 2000). PPAR-γ agonists have antiangiogenic, antiproliferative and pro-differentiation effects (Tontonoz P. et al, 2008). TZDs are found to reduce proliferation of prostate, colon and breast cancer cells in humans (Murphy GJ et al, 2000).

1.4.5. Mechanism of action of PPAR-γ in anticarcinogenecity (Sun J. et al, 2023)

Anticancer effects of PPAR-γ are well accepted now. In a study in 2019, it was found that those patients who had increased expression of PPAR-γ had better prognosis for colorectal cancer. PPAR-γ prevents colorectal cancer incidence by regulating expression of cellcycle regulators as well as cell differentiation. In case of liver and cervical cancer, expression of PTEN (tumor suppressor gene) is upregulated and PI3K (signaling) pathway is downregulated as well as decreases proliferation abilityof cancer cells. Progression of cancer cell is inhibited by activated PPAR-γ which ablates cyclin D1andresults in cell arrest. In same way, activated PPAR-γ stimulates transcription of gene p21 and induces arrest of cell cycle in G0/G1 phase, in gastric and colorectal cancer cell. In rat model, PPAR-γ upregulates PTPRF (gene) and thus poses inhibitory effect on breast cancer cell growth.

Stromal cells like cancer associated fibroblasts (CAF) andcancer associated adipocytes (CAA) are important for maintenance of tumor microenvironment (TME), as they provide nutrients for growth. Immune cells are activated by CAF inside TME and activation as well as deposition of ECM (extracellular matrix) is characteristic feature of cancers. PPAR-γ remodels CAF in several ways, and its expression is up regulated in colorectal cancer and breast cancer, converting CAFs into energy providing part for growth of cancer. PPAR-γ stimulates mesenchymal cells to form adipose cells which further convert into CAA. Healthy fat cells produce energy and chemokines that affect tumor nature. In BC, upon removal of PPAR-γ inside CAA decreases expression of BRCA1 and increases cancer. In another *in vivo* study of breast cancer, PPAR-γ differentiates cancer cells into adipose cells and causes apoptosis of adipose cells, thus stopping breast cancer.

In epithelial cancers, mostly Wnt/β-catenin pathway is found disrupted that resultinto increased synthesis of enzymes of aerobic glycolysis. PPAR-γ after activation results in stoppage of β-catenin pathway, and gets inactivated after Wnt/β-catenin pathway gets activated. Most cancers are developed oftenly by upregulation of Wnt/β-catenin pathway and downregulation of PPAR-γ. Wnt ligands translocates β- catenin into nucleus and binds to target genes like COX-2, c-Myc and pyruvate dehydrogenase kinase (PDK) whereas downregulation of PPAR-γ is related to increased expression of Wnt/β-catenin (pathway). MCT-1 secretes extra cytoplasmic

lactate. Thus, PPAR-γ inhibits MCT-1 and PDK1, resulting into Wnt/β-catenin pathway activation ineffective. PPAR-γ after activation promotes cell differtiation, cell cycle and apoptosis whereas Wnt/β-catenin pathway after dowregulation decreases oxidative stress and inflammatory factors like IL-6, IL-8 and TNF-α, TGF-β. Thus, metastasis in liver cancer is decreased.

1.4.6. Significance of PPAR-γ agonists as anticarcinogen

PPAR-γ agonists like TZD have been already well studied for the treatment of diabetes to lower blood glucose. It has also been used to resist cerebrovascular and cardiovascular diseases. This class of drugs is also utilized against cancer treatment as these drugs are involved in induction of apoptosis, proteasomal degradation for induction of cell cycle arrest, decrease in intracellular Ca2+, inhibition of hormonal receptors, reducing macrophage activation, decreasing cellgrowth by decreasing expression ofBcl2, b-FGF, c-Mycand VEGF (Dumasia, R. et al, 2005).

Ciglitazone initially found to stop cancer cell growth by decreasing Ca_2+ inside BC cells (Kwon, C. H. et al, 2009). In apoptosis, ciglitazone was found to act against Bcl-2 as well as Bcl-xLand found to improve internal apoptotic activity inside cells of prostate cancer (Yang, C. et al, 2007). Ciglitazone decreases apoptosis by stopping FLIP, an apoptosis inhibitor protein (Plissonnier, M. L. et al, 2011). Pioglitazone activates PTEN and results in ER degradation. It also causes /inhibition of aromatase by BRCA1 and PGE2 signaling pathway. Thus, pioglitazone is also capable of preventing growth of BC (Chi, T., et al, 2021, Kelloff, G. J. et al, 2006).

Likewise, in HCC cell lines, rosiglitazone activates PPAR-γ which binds onto PTEN promoter for increasing expression of PTEN as PTEN acts as inhibitor of PI3K/AKT/mTOR cell proliferation signaling pathway, thus stops cancer. When it binds to PPAR-γ, another signaling pathway MAPK/ERK is inhibited by rosiglitazone. PPAR-γ after activation by rosiglitazone inhibits phosphorylation of ERK, thus stopping the growth of BC cells. Rosiglitazone promotes cell death in cells of cancer too. In pancreatic cancer, rosiglitazone resultsin apoptosis by increasing expression of Bax acceleration factor. In colon cancer, activated PPAR-γ by rosiglitazone is found to improve treatment by inhibiting COX-2. In breast cancer, rosiglitazone activated PPAR-γ decreases expression of NHE1 acting as regulator of homeostasis, which makes tumor cells sensitive to death, histopathological analysishas confirmed this finding (Luongo, F., et al, 2019, Dang, Y. F. et al, 2018, Chi, T., et al, 2021).

Efatutazone, being a novel PPAR-γ agonis tupregulates PTEN and PPAR-γ expression that makes Akt pathway inactive. This consequently causes inhibition of EGFR-TKI pathway in adenocarcinoma of lung. EGFR-TKI pathway works along with LXRα, member of nuclear receptor of another class, is considered as important target against different cancers. It is found in phase I clinical trial thatefatutazone when given orally, increases efficiency of paclitaxel during treatment of thyroid cancer. In addition to this, balaglitazone, another TZD, is found to increase expression of PTEN and PPARγ, causing partial reversal of multidrug resistance caused by P-glycoprotein, inside doxorubicin- resistant, K562/DOX (human myeloid leukemia) cells (Smallridge, R. C. et al, 2013, Ni, J., Zhou et al, 2017, Hong, F. et al 2018, Sun, J. et al, 2023, Chi, T., et al, 2021).

Combined therapy can also be given, like ciglitazone and cisplatin (chemotherapy drug) can be given together for improved treatment of ovary cancer in human (Yokoyama, Y. et al, 2011). TGZ (Thioglitazone) and RXRα ligand increases growth inhibitory effect and apoptosis on breast and gastric cancer cell lines. Breast and ovarian cancer cell lines do not show any effect for conventional therapy, then combined therapy of TGZ and TNF-associated apoptosis-inducing ligand (TRAIL) isused to get synergistic effects for apoptosis (Sun, J. et al, 2023). In advanced stage of hepatocellular carcinoma, chemotherapy gets failed due to resistance for 5-fluorouracil (5-FU), whereas PPAR-γ activated by rosiglitazone upregulates PTEN expression and downregulates COX-2 expression, resulting into increase in sensitivity for 5-FU in HCC cell lines, against cancer (Mrowka, P. et al 2020, Dang, Y. F. et al, 2018). Furthermore, rest of the drugs like aspirin, lovastatin and tamoxifen (an estrogen modulator) had been reported as synergistic drug for cancer along with TGZ against lung, thyroid, cervical, glioblastoma and breast cancer (Sun, J. et al , 2023, Chi, T., et al, 2021).As PPAR-γ receptor is actively involved in combating cancer, but its activation by full synthetic agonists like TZDs are associated with several drawbacks like fluid retention, weight gain and liver problems, due towhich these drugs are not advisable. So, natural PPAR-gamma agonists which act like partial PPAR- gamma agonists will activate PPAR-γ receptor partially and will be associated with fewer side effects (Amano, Y. et al, 2012). Thus, natural PPAR-γ agonists are very effective and admirable therapeutic option against cancer. Different PPAR-γ agonists like gallotanin have anticarcinogenic effect by activating PPAR-γ receptor (Wang L. et al, 2014). Resveratrol, isoflavone, curcumin, nabiximols, cannabidiol and medical cannabis are also natural PPAR-γ agonists which are found to have potential against different types of cancer (Wu, L. et al 2020).

1.4.7. PPAR-γ agonists for ameliorating TNBC

It is observed that PPAR-γ agonists can be used for treatment of TNBC. Epigenetic depression by HDAC inhibitors converts PPAR-gamma receptor into druggable target against TNBC (Loo, S. Y. et al2021). PPAR-γ ligand induces expression of Annexin A, which further determines chemotherapy response by deubiquitination of death domain kinase RIP in TNBC (Chen, L. et al, 2017). Different PPAR-γ agonist compounds have been found to be useful against TNBC. Epoxy- Eicosatrienoicacidderegulates FABP4/FABP5 pathway, associated with it, to act as therapeutic approach against metastatic TNBC (Apaya, M. K. et al, 2020). Xianlinglianxiafang is found to inhibit growth and metastasis of TNBC by activating PPAR-gamma receptor (Yang, X. et al, 2023). VSP-17, a novel PPAR-γ agonist also found to suppress metastasis in TNBC by upregulating the expression of E- Cadherin (Wang, Y. et al, 2018). Thus, PPAR-γ agonists can act as very promising approach against TNBC. However, still there is a research gap for discovering potent, natural PPAR-γ agonist against TNBC, which must be fulfilled.

1.4.8. Natural PPAR-γ agonists against TNBC

There are few natural PPAR-γ agonists which has been used as a therapeutic option against TNBC, like Resveratrol, a PPAR-γ agonist and a natural compound obtained from grapes is found to have therapeutic effect against TNBC (Shindikar A. et al, 2016). Another PPAR-γ agonist (−) EGCG (epigallocatechin 3- gallate) which is a major component of green tea, found to have anticancer potential against different types of cancers. They reviewed potential of EGCG as a therapeutic option against TNBC (Bimonte S. et al, 2020). Carnosol, obtained from ethanolic extract of rosemary, is alsoa PPAR-γ agonist, is found to induce apoptosis and autophagy in TNBC (Al Dhaheri, Y. et al, 2014). Luteolin was also found to have anticancer effect against TNBC as it suppressed transition of epithelial cells to mesenchymal cells and it was also found to decrease migration of TNBC cells as it inhibits YAP/TAZ activity (Cao D. et al, 2020). These above mentioned PPAR-γ agonists are reported in the article of Limei Wang (Wang L. et al, 2014). However, there is extreme need to explore more natural PPAR-γ agonists against TNBC, as natural compounds are safer therapeutic option with profound effectiveness.

CHAPTER 2
REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1-PPAR-γ receptor in breast cancer

Woo C. et al, 2011 observed that thymoquinone exerts anticancer effect on breast cancer cells by activation of PPAR-γ receptor. When thymoquinone was given along with doxorubicin and 5-fluorouracil, then it increases cytotoxicity. Thymoquinone found to decrease the expression of Bcl-xL, Bcl-2, and survivin inside the breast cancer cells. Thus, Thymoquinone can act as PPAR-γ agonist also. Jiang W. et al, 2003 examined transcription level of PPAR-γ receptor and its coactivator PGC-1 in a cohort study with breast cancer patients. They observed that although the level of expression of PPAR-γ receptor in breast cancer cells was lower than normal tissues, but its expression was higher in non invasive breast cancer cell line than invasive cell line. However, it was found that level of expression of PGC-1 does not correlate with the expression of PPAR-γ receptor for nodal development and grade of breast cancer. Kotta-Loizou, I. et al, 2012 concluded that as PPAR-γ receptor was found overexpressed in certain cancers including breast cancer, which suggests its important role in cancer developments. Natural and synthetic ligands of PPAR- γ receptor found to increase growth inhibition, causes differentiation and apoptosis in cancer cells. Bonofiglio, D. et al, 2009 observed in an experiment that PPAR-γ receptor induces apoptosis in breast cancer cells of humans by activating Fas ligand gene promoter. Ditsch, N. et al, 2012observed presence of RXR and PPAR-γ receptor in breast cancer and its associations with clinical symptoms.

Fenner, M. H. et al, 2005 observed that PPAR-γ signaling is found involved in different kind of cancers like prostate, colon, liposarcoma etc. PPAR-γ ligands are found to decrease cell proliferation, angiogenesis, invasion and increase apoptosis. They explored mechanism which is used by PPAR-γ agonist to be used against breast cancer. Kim KY et al, 2006 investigated role of PPAR-γ agonists against breast cancer. They found that rosiglitazone and KR-62980 had antiproliferative effect via apoptosis. They observed that PPAR-γ agonists increases PTEN expression and decreases Akt phosphorylation. They found that PTEN is critically involved in anticancer role of PPAR-γ activation. Augimeri G. et al, 2020 reviewed again the role of PPAR-γ agonists in ameliorating breast cancer. As PPAR-γ receptor upon activation is involved in apoptosis, cell proliferation and invasion in breastcancer, so both synthetic and natural PPAR-γ agonists were explored to know the possible outcomes. Memisoglu A. et al, 2002 observed that although estrogen is primarily involved in breast carcinogenesis, but PPAR-γ upon activation decreases the transcription of aromatase enzyme which is involved in estrogen

biosynthesis. PPAR-γ agonists increase differentiation and decreases markers of breast cancer malignancy. They investigated in a case control study the association of breast cancer with $Pro^{12}Ala$ *PPAR-γ* polymorphism. They found that $Pro^{12}Ala$ *PPAR-γ* polymorphism is not associated with wight gain or body mass.

Pignatelli M. et al, 2003 observed that increase in expression of BRCA1 gene is related with anticarcinogen activity. They found that two PPAR-γ ligands; 15-deoxy-$\Delta^{12,1}$-prostaglandin J_2 (15dPG- J_2) and rosiglitazone increases BRCA1 expression by activating PPAR-γ receptor. Pon CK. et al, 2015 found that insulin like growth factor binding protein 3 is involved in PPAR-γ mediated inhibition of breast cancer. IGFBP-3 and PPAR-γ ligands 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, or rosiglitazone separatelystopped the growth of MCF-7, MDA-MB-468 and MDA-MB-231 breast cancer cells. However, when both PPAR-γ agonists given alone were not that much effective as when given together. Jiang W.G. et al,2000 found that PPAR-γ upon activation transmits the action of gamma linolenic acid inside breast cancer cells. Gamma linolenic acid regulates cell attachment and induces cytotoxicity in breast cancer cells. They showed that the action of gamma linolenic acid is due to phosphorylation and translocation ofPPAR-γ. Laxmi A. et al, 2023 elaborated that how phytochemicals are beneficial for epigenetic post- translational histone modifications associated with breast cancer which are involved in changing structure of chromatin and regulating the transcriptional activity of specific oncogenes which are crucial for causing breast cancer.

2.2- Different therapeutic approaches for triple negative breast cancer

Schmid P. et al, 2022 explained that the usage of Pembrolizumab in neoadjuvant chemotherapy for patients of early triple negative breast cancer reduces occurrence of metastasis. They talked about event free survival of patients with use of pembrolizumab. This shows its potency. Bardia A. et al, 2021 observed that Sacituzumab govitecan which is an antibody-drug conjugate targets human trophoblast cellsurface antigen 2, expressed in mostly all breast cancer cells. Metastasis is most serious problem of triplenegative breast cancer. They evaluated Sacituzumab govitecan with the single chemotherapy drug among vinorelbine, eribulin, gemcitabine. The end point was considered as progression free survival. Ghanem,
A. et al 2023 observed that *Rumex vesicarius* L. which has antioxidant potential, can be used toenhance the effectiveness of sorafenib, a drug used for treating triple negative breast cancer, alone and in combination, on triple negative breast cancer cell line MDA MB-231 to know the underlying mechanism by in vitro and insilico methods. The found that *Rumex vesicarius* L.

decreases the expression of JNK, mTOR and BCl2 genes and simultaneously increases the expression of p21 gene to increase the efficacy of sorafenib.

Yang F. et al, 2023 observed that ferroptosis can be used as new insight in the treatment of triple negative breast cancer. They attached their large data i.e. n=465 as a cohort study for developing ferroptosis atlas. They discovered that triple negative breast cancer patients had different phenotypes for metabolism and metabolic pathways related to ferroptosis. GPX4 inhibitors were used to induce ferroptosis. They verified that inhibition by GPX4 induced ferroptosis in tumor and simultaneously increased immunity against tumor too. They also observed that combination of both GPX4 and anti PD1 had better therapeutic capability than monotherapy. Higher GPX4 expression was found related with bad prognosis in cohorts of immunotherapy and lower cytolytic score. Over all they mentioned landscape of ferroptosis for triple negative breast cancer and showed innovative combination immunotherapy strategy for LAR tumors. Ogier du Terrail, J. et al, 2023 found that the use of neoadjuvant chemotherapy is the current standard of treatment for nonmetastatic tumors but the efficacy of treatment varies among the patients. The reason of this heterogeneity is not properly understood. Thus, they investigated the usage of machine learning using clinical information and slide images, to predict the histological response for neoadjuvant chemotherapy at the time of diagnosis in early triple negative breast cancer patients. Biasness in small scale study can be overcome by using federated learning in multicentric study.

Kong, X. et al, 2023 explained in their review that severe side effects and toxicity of chemotherapy drugs and formation of resistant tumor cells in triple negative breast cancer is a serious problem for therapeutic strategies. So, they explained nanoparticles as a system for drug delivery to improve the efficacy of treatment. They told that combining nanoparticle drug delivery system with photothermal ablationtherapy and found marked reduction in toxic effect of chemotherapy drugs. They also explained decreasein growth of tumor cells and activation of immune system. Nanoparticle delivery changes the pharmacokinetic roles of chemotherapy drugs and reduces toxicity upto a significant level by more targeted delivery of drug. Cruz-Gregorio, A. et al, 2023 observed that polyphenol α-Mangostin, which has anticarcinogenic effect in several cancer models and has the capacity to affectprooxidant andinflammatory state in different disease models, can be used as a putative treatment against TNBC. They found that oxidative damage, oxidative stress and the redox state are especially related to development and treatment of cancer. Their study focused on to know the effect of α-Mangostin on mitochondrial metabolism, redox state and apoptosis in 4T1 breast cancer cells. In this study,

they observed that α- Mangostin decreases both concentration and enzyme activity of catalase and increases both glutathione disulphide, oxidized proteins and reactive oxygen species, which shows thatα-Mangostin, does oxidative damage. They also found that α-Mangostin promotes dysfunction of mitochondria and decreases concentration of oxidative phosphorylation units of I, II, III and IV complexes of mitochondria. Thus induction of mitochondrial dysfunction and oxidative damage by α-Mangostin results into apoptosis of 4T1 cells and finally into death of 4T1 cells.

2.3- PPAR-γ receptor and its different agonists for triple negative breast cancer

Loo, S. Y. et al, 2021 showed that increased PPAR-γ expression is related to betterment for triple negative breast cancer, but histone deacetylase over expression restricts antiproliferative action of PPAR-γ agonists. So HDAC inhibitors can be used as associative therapy for cancer. Thus they observed that combination therapy of HDAC inhibitors and PPAR-γ agonists works together in better way than alone.

Shen, S. J. et al, 2020 found that in triple negative breast cancer, PPAR-γ receptor is inhibited by microRNA-27b-3p, due to which metastasis and tumor growth is increased. Thus, they observed that microRNA-27b-3p increases progression of triple negative breast cancer by inhibiting PPAR-γ receptor. So, they concluded that PPAR-γ can be considered as potent molecular target and microRNA-27b-3p can be considered as efficient prognostic marker for triple negative breast cancer. Yang, X. et al, 2023observed that xianlinglianxiafang inhibits proliferation and metastasis and increases apoptosis in triple negative breast cancer by activation of PPAR-γ and AMPK signaling pathway, and prolong the survival time of patients. Thus they observed that PPAR-γ /AMPK signaling is mainly the cause of efficacy of xianlinglianxiafang in treating triple negative breast cancer. Xu X. et al, 2021 found that VSP-17 inhibits EMT process by involving pathway of PPAR-γ /AMPK signaling, and thus suppresses invasion and migration of triple negative breast cancer. VSP-17 is a novel PPAR-γ agonist. Theybasically found out the mechanism of action of VSP-17. Their study was focused on how PPAR-γ /AMP pathway activates AMPK and results in inhibition of effects of VSP-17 on cells of triple negative breast cancer. Clearly, it involves PPAR-γ.

Apaya, M. K. et al 2020 observed that protein expression of FABp4, FABP5 and CYP2C19 is increased in case of TNBC and very critical for progression of metastasis in TNBC. They also found thattranslocation of FABP4 and FABP5inside the nucleus is assosciated with EET (epoxy-eicosatrienoic acid) and PPAR- γ causes growth of TNBC cells and metastasis. Most

specifically they uncovered new mode of action and efficacy of doxorubicin and a phytochemical 1, 2-di-*O*-α-linolenoyl-3-*O*-β- galactopyranosyl-*sn*-glycerol (dLGG) against lung metastasis and TNBC by deregulating levels and dynamics of EET/FABP. So, this study provided a novel method of combating TNBC by focusing on metastatic signaling network associated with FABP/EET/CYP. Huang, X. et al, 2023 examined the mechanism of anticancer effect of asiaticoside against TNBC. In this study, they found that asiaticoside stopped expression of TGF-β1 and phosphorylation of SMAD2/3 in TNBC cells and thus disturbing signaling of TGF-β/SMAD. In this way asiaticoside stopped invasion, migration and epithelial mesenchymal transition in TNBC cells by decreasing signaling of TGF-β/SMAD. They identified PPAR-γ as a potential target of asiaticoside. Asiaticoside increases expression of PPAR-γ and theknockdown of PPAR-γ attenuated the therapeutic effect of asiaticoside. However, overexpression of PPAR-γ decreased transcription of P2X purinoceptor
7. The comeback of P2X purinoceptor 7 also reverses the therapeutic effect of asiaticoside. So, these results explained that asiaticoside increases PPAR-γ expression which blocks P2RX7-mediated signaling of TGF- β/SMAD, thus decreasing epithelial mesenchymal transition in TNBC cells.

2.4- PPAR-γ receptor in clinical scenario

Ding, Y. et al, 2020 summarized the research related to involvement of PPAR-γ for protection from CI/RI (Cerebral ischemia reperfusion injury). They explained the mechanism involved at both cellular and molecular level to bring changes. They also talked about inhibition of toxicity of amino acids, antioxidative stress, reduced calcium ion overload, anti-inflammation, maintenance of blood brain barrier, inhibition of activation of microglia, anti-apoptosis, promotion of neurogenesis and angiogenesis. Katoch S. et al, 2022 advocated on the basis of available literature that PPAR-γ receptor can act as molecular target for hepatocellular carcinoma. They also talked about clinical and experimental scenario while usage of natural and synthetic PPAR-γ agonists against hepatocellular carcinoma. They also elucidated role of PPAR-γ in pathogenesis of hepatocellular carcinoma. Zahr, T. et al, 2023 showed in an experiment on mice that PPAR-γ upon activation leads to counteraction of metabolic dysfunction caused by age related onset of atherosclerosis. They also advocated that deacetylation of PPAR-γ provides protection from onset of atherosclerosis caused due to age factor. Rashid, M. M et al, 2022 conducted an insilico, invitro and invivo experiment to investigate the effect of Kattosh stem extract on the damage caused to liver, kidney and pancreas, induced by Streptozotocin. In insilico study, they interacted kattosh stem extracted

phytocompound with the alpha amylase enzyme, AMP activated protein kinase and PPAR-γ receptor for verification of invivo results. In results they found highest affinity of Kattosh stem extract for PPAR-γ receptor for attenuating the streptozotocin induced liver, pancreas and kidney damage.

Omoboyowa, D. A. et al, 2023 used computational analysis for Abrus Precatorius by docking, MD simulations, quantum chemical calculations, ADMET studies to prove that this phytochemical as a modulator of PPAR-γ. They observed that this phytochemical Abrus Precatorius got higher binding energy than reference compound acarose. Quantum chemical calculations also revealed that this compound has better chemical reactivity and bioactivity with better intramolecular charge transfer as electron acceptor and electron donor. Yin, L. et al, 2022 reviewed that PPAR-γ has influential role against atherosclerosis by increasing repression of monocytes, cholesterol efflux, transformation of monocytes into macrophages and by decreasing migration and growth of vascular smooth muscle cells. They discussed that post translational modification of PPAR-γ leads to activated and increased role of PPAR-γ agonists against the atherosclerosis, which is considered to be the major cause of cardiovascular diseases.

2.5- PPAR-γ agonists for triple negative breast cancer

Chen L. et al, 2017 established that triple negative breast cancer subtypes have high content of Annexin A1. PPAR-γ ligands deubiquitinates death domain kinases RIP and provide chemotherapy response. This all is due to increase in expression of Annexin A1, caused by PPAR-γ agonists. Hence, they elaborated the importance of usage of PPAR-γ agonists against triple negative breast cancer. Wang Y. et al, 2018 designed a PPAR-γ agonist VSP-17 which increases the expression of E-Cadherin and decreases the increase of metastasis in triple negative breast cancer cells. VSP-17 is a novel PPAR-γ agonist evidencedby activity of PPAR-γ reporter gene and upregulation of CD36 expression. In triple negative breast cancer cells MDA MB-231 cells, it clearly suppressed metastasis in liver by increasing expression of E- Cadherin. Jiao, X. et al, 2020 observed the effect of pioglitazone as PPAR-γ agonist in breast cancer. They tested pioglitazone by Scratch, cell viability and transwell assay to show inhibitory effect of pioglitazone on breast cancer cells using PPAR-γ as receptor and JAK2/STAT3 pathway. They also examined antiproliferative and anti- invasive effect of pioglitazone against breast cancer using nude mice model.

Malaviya, A. et al, 2013 investigated the effect of γ-Tocotrienol in association with PPAR-γ

agonist or antagonist against breast cancer. γ-Tocotrienol is vitamin E, known to possess anticancer potential. They explained that combined treatment of γ-Tocotrienol and PPAR-γ ligand results into decreased PPAR-γ expression and increased mitogenic activity and decreased PI3K/AKT pathway. Burstein, H. J et al, 2003 evaluated therapeutic potential of Troglitazone, a PPAR-γ agonist for metastatic refractory breast cancer. They considered patients having advanced breast cancer either with ER positive tumor or with ER negative tumors and treated them with trolglitazone 800 mg for 6 months to determine betterments in number of patients. They examined toxicity, serum tumor markers and tumor response after treatment to know about changes in tumor differentiation. In result, serum tumor markers were found elevated. So, they found that troglitazone has little potency by activating PPAR-γ for treatment against metastatic refractory breast cancer.

2.6- PPAR-γ agonists in other diseases

Colle, R. et al, 2017 evaluated the efficacy of PPAR-γ agonists as antidepressants in a clinical trial against major depression, by estimating the changes in biomarkers of inflammation and metabolism. 448 patients were included in the trial, out of which 209 were given PPAR-γ agonists Pioglitazone or troglitazone, either alone or along with conventional treatments for 6-12 weeks. They found that improvement was observed in depression score along with improvements in biomarkers for glucose tolerance test, insulin resistance, inflammation etc. They studied total 21 biomarkers and found that PPAR-γ agonists may have antidepressant property but more assessment is required for confirmation. Omeragic, A. et al, 2019 observed that PPAR-γ agonists exhibit antiviral and anti-inflammatory effects

in neurological disorders. HIV-1 associated neuro cognitive disorders currently have no treatments, so there is urgent urge to find one. They targeted PPAR-γ to treat inflammation related with HIV associated neurocognitive disorders. They showed that treatment of pioglitazone and rosiglitazone on mouse glial cells reversed oxidative stress and inflammatory genes, when given intraperitoneally. They concluded that PPAR-γ agonist treatment decreases p24 protein burden inside the brain, thus they act as anti- inflammatory and antiviral, and can act as promising drug for HIV-1 associated neurocognitive disorders. Stavniichuk, A. et al, 2020 developed a soluble dualepoxide hydrolase inhibitor-PPAR-γ agonist RB394, (sEHi/PPAR-γ) and investigated its capability to treat renal fibrosis (which ultimately leads to kidney damage), in mouse UUO (unilateral ureteral obstruction) model. The efficacy of RB394 was compared to sEH inhibitor, rosiglitazone a PPAR-γ agonist. They ultimately concluded in

this experiment that RB394 has capacity to treat renal fibrosis by reducing oxidative stress, vascular injury, renal inflammation and tubular injury. Thus, RB394 has great potential to act as a therapy for chronic kidney disease and renal fibrosis.

Kumar, B. P. et al, 2020 reviewed and explained that PPAR-γ is involved in several important pathways, and also considered as promising receptor for neurodegenerative diseases also. They explained about evidences which tell that PPAR-γ agonists have potential to ameliorate neurodegenerative diseases as they have protective effect against it. This neuroprotective effects comes from interaction of PPAR-γ with coactivator of PPAR-γ receptor (PGC-1alpha). They explained different studies which tell that PPAR-γ activation can lead to neuroprotective effect and PPAR-γ activation is done by PPAR-γ ligands. So, thereis great scope for discovering or synthesizing new drugs which act as PPAR-γ agonists to act as neuroprotective agent. Sharma, V. et al, 2022 reviewed and discussed that PPAR-γ is involved in the pathophysiology of kidney diseases. They advocated the efficiency of natural PPAR-γ agonists for treating different kidney diseases like diabetic kidney disease, acute kidney injury, hypertension nephropathy, obesity induced nephropathy and IgA nephropathy. They also discussed that although PPAR-γ can be potentially activated by synthetic PPAR-γ agonists for treating kidney diseases but its usage must be avoided due to several side effects of synthetic PPAR-γ agonists. So, they mainlyemphasized on the usage of natural PPAR-γ agonists for treatment of kidney diseases.

2.7- Mechanism of PPAR-γ agonist's pathway of action against cancer:

In several experiments it is found that PPAR-*γ* acts as tumor suppressor because it is found to decrease tumor growth, invasiveness and proinflammatory cytokine production in several cancers like prostate, lung, colon, pancreatic and breast cancer, after activation of PPAR-*γ* /RXR signaling. Tumor suppressive function of PPAR-*γ* also include metabolic reprogramming, essential biochemical hallmark of cancer viability (Hernandez-Quiles, M. et al, 2021). Insulin like growth factors (IGF) system has role in development and growth of cancer as suggested by experimental and epidemiological evidences. Increased plasma concentration of IGF-1 is found to be linked with several malignancies. Patients with increased serum IGF-1 levels are shown to be at high risk to get premenopausal breast cancer, lung cancer, prostate cancer, colorectal cancer, bladder cancer and endometrial cancer in comparision to people who have lower levels. In transgenic animals, forced expression of IGF-1 and IGF-2 is linked with increased cancer growth. IGF system overactivation or dysregulation leads to constitutive

activation of two intracellular signaling mechanism namely, the PI3K/mTOR and the ERK1/2 signaling pathways. PTEN, a lipid phosphatase capable of inhibiting PI3K pathway, is often inhibited in human cancers which leads to the abnormal signaling of the IGF system and eventually cancer development. PPAR-γ activation by PPAR-γ agonists results in induction of apoptosis. In lung cancer, apoptotic response is initiated by troglitazone in PPAR-γ and ERK1/2 dependent manner. Troglitazone decreases Bcl-2 (antiapoptotic protein) levels and causes colocalization and nuclear accumulation of PPAR-γ and ERK1/2. In addition to apoptosis, PPAR- γ overexpressed in many human cancers. It is observed that TZD exposure for 24 hours causes G_0/G_1 cell activation decreases tumor growth by inhibiting cancer cell proliferation by inducing cell cycle arrest. Cyclins are proteins which regulate cell cycle and specifically activate cyclin-dependent kinases (CDKs). As they are involved in control of cell cycle, cyclins are potential oncogenes. Cyclin D1 is found

cycle arrest. Treatment with TZD, decreases levels of cyclin D1 and also decreased growing cell nuclear factors like Cdk4p and Rb, and increased p21 and p27 which are cyclin-dependent kinase inhibitors. In thyroid anaplastic cancer, rosiglitazone was found to induce cell growth arrest by increasing p21 and p27 (CDK inhibitors), decreasing expression of cyclin D1 and by activating Rb protein. Another mechanism by which PPAR-γ agonists act against tumor is by inducing cell differentiation. In breast cancer cell culture, PPAR-γ agonists resulted in extensive accumulation of lipid and changes in expression of epithelial genes related with more differentiated and less malignant state. Another mechanism by which PPAR-γ agonists results in inhibition of tumor initiation and progression is by promoting angiogenesis and by decreasing inflammation of tumor microenvironment (Belfiore, A. et al, 2009).

It was also observed that in patients of non small cell lung carcinoma, Rosiglitazone which is a PPAR- γ agonist increased antitumor activity of gefitinib by increasing expression of PPAR-γ and PTEN. PTEN changes phosphatidylinositol 3-kinase (PI3K) pathway involved in cell proliferation. Rosiglitazone decreased growth of A549 cells treated with gefitinib in dose dependent manner (Lee, S. Y. et al, 2006).

CHAPTER 3
HYPOTHESIS

HYPOTHESIS

The detailed literature perusal has revealed that researchers around the globe are trying tirelessly to get optimal therapeutic option for TNBC. TNBC has limited treatment options due to absence of otherwise present receptors in breast cancer. Chemotherapy, the remaining mainstay, itself has several side effects. So, PPAR-γ receptor, found expressed and actively involved in combating different cancers need to be explored more for TNBC too. PPAR-γ is of considerable therapeutic and academic value since beginning of its discovery in 1990s. Much has been revealed due to worldwide investigations about the involvement of PPAR-γ in diabetes, neurological disorders, atherosclerosis, kidney disorders, various kinds of cancers etc. While PPAR-γ agonists are being investigated for several diseases, but approval for treating diabetes is given to full PPAR-γ agonist called as thiazolidinediones. However, thiazolidinediones (rosiglitazone) are found to cause serious life threatening damages like liver damage, bone fractures, fluid retention causing heart attacketc and even found to cause cancer in rats indifferent studies. Thus, rosiglitazone was withdrawn from the market. Keeping this scenario in mind, there is urgent need to find naturalPPAR- γ agonist which can act as partial PPAR-γ agonist, and thus will exert no or fewer side effects.

Natural compounds are of pharmaceutical value since Vedic era. Nutraceuticals is nowadays of utmost craze for researchers as these are natural compounds having therapeutic importance and less or no side effects. Thus, the proposed study was conducted to find natural, potent PPAR-γ agonist, having anticancer potential for treating TNBC. For this, firstly insilico study was conducted using established PPAR-γ agonists as a query to find new one on the basis of structure based similarity searching,
/molecular docking and ADMET profiling. Then searched compounds were further screened and validated by in vitro and in vivo methods. This is the first study of its type to exploit all three validation methods, insilico, invitro and invivo together, to evaluate the potency of any natural compound against TNBC. In this research study, we have explored different natural compounds using insilico, invitro and invivo methods to validate them as a potent, natural PPAR-γ agonist having potential to treat TNBC.

Null hypothesis: PPAR-γ agonist is not effective for treating TNBC. Alternative hypothesis: PPAR-γ agonist is effective for treating TNBC.

Interpretation: According to our obtained results, we reject our null hypothesis and accept alternative hypothesis, as selected PPAR-γ agonists were found effective for treating TNBC.

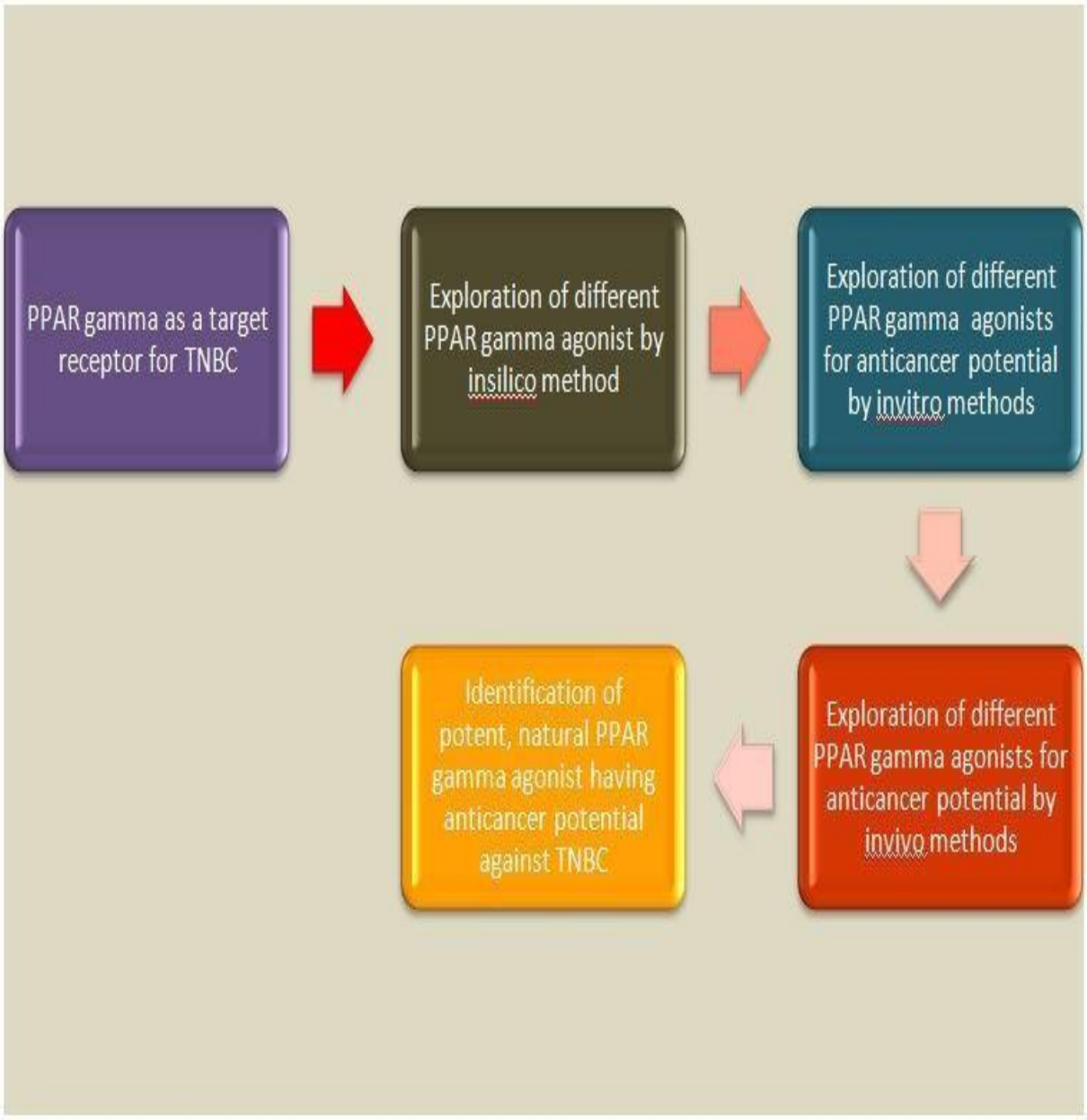

Figure 3.1: Showing hypothesis of PhD research work

CHAPTER 4

AIMS AND OBJECTIVES

AIMS AND OBJECTIVES

Aim:

PPAR-γ is considered as target for several diseases including cancer. Synthetic, full PPAR-γ agonists like thiazolidinediones have been found to combat cancer by decreasing angiogenesis, cell proliferation and metastasis, but they cause serious life threateningside effects like hepatic damage, fluid retention, bone damage etc. Moreover, few thiazolidinediones have found to even increase cancer incidences. Thus, natural, partial PPAR-γ agonists must be searched and explored for their efficiency to combat cancer as they confer less or no side effects. Very few natural PPAR-γ agonists have been explored yet for their anticancer potential against TNBC. In this study, we aim to search and explore natural, potent PPAR-γ agonists having anticancer potential against TNBC utilizing insilico, invitro and invivo methods. To achieve this aim, following objectives were designed, which are given as follows:

Objectives:

1. Similarity search based data mining of known PPAR-γ agonists and molecular docking against PPAR-γ receptor to identify potent natural lead compound.
2. In vitro study to check the effect of identified compounds on cell viability of triple negative breast cancer cells, their PPAR-γ agonistic activity and antioxidant potential.
3. In vivo study by CAM assay, using novel identified PPAR-γ agonists to check their ability to decrease angiogenesis and cell invasion.

CHAPTER 5

MATERIALS AND METHODS

MATERIALS AND METHODS

5-Chemicals:

The analytical grade chemicals used in experimentations are: Silymarin and Hesperidin (both 98.9% pure) were purchased from Biomall, India, with CAS no. 65666-07-1 of Silymarin and CAS no. 520-26-3 of Hesperidin. Doxorubicin was procured from Kach Biotech, India. Cancer cells MDA MB-231 were purchased from National centre of cell science, Pune, India. Primary antibody for western blotting, PPARγ Antibody (E-8): sc-7273 and Actin Antibody (2Q1055): sc-58673 and secondary antibody, Goat anti-mouse IgG-HRP: sc-2005 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primers of real time PCR were synthesized by Midland Certified Reagent Company Inc.

5.1- In Silico study:

This is the study of interaction of phytochemical or ligand with the receptor inside the computer to summarize or visualize the potency of that compound to act as a drug against a particular disease. For this type of study to pursue, it is essential to know either target or ligand. All the softwares used in *insilico* study were already calirated, and standardization, calibration and validation of the different techniques used was done by using PPAR-γ x-ray co-crystallized ligand (cercosporamide derivative) as standard for validation.

5.1.1- Data collection and preparation

The three-dimensional structure of PPAR-γ (PDB ID: 3V9T) was downloaded from the RCSB Protein Data Bank (PDB).47 natural PPAR-γ agonists were found reported in review article of Leimie Wang (Wang L. et al, 2014), which acted as query compounds for structure-based similarity searches in PubChem database. Structure of all these 47 compounds is given in table 7.

5.1.2- Datamining and similarity searches

Datamining is an *insilico* method of searching ligands in databases using a query ligand or compound. Data mining in PubChem database was done employing 47 natural PPAR-γ agonists, reported in published literature, as a query sequence to search similar PPAR-γ agonists of same structure. To accomplish this structure based similarity search, smile notation of query compounds were employed to filter compounds having 80% similarity in structure in PubChem. 2-Dimensional structure based search was done based on fingerprint Tanimoto

coefficient. For every query sequence, 4 similar structures were searched. Molecular similarity is routinely used for drug designing and discovery. It is based on the notion that if two molecules are structurally similar then their physical and biological properties will also be similar. Tanimoto coefficient is widely used method for similarity measurement (Jagannathan, R. 2019). As it is a 2D similarity method so it relies only on the 2D structural information, thus it is an efficient, fast and very popular similarity search method. As query compounds are PPAR- γ agonists, so when these compounds are searched in PubChem database, structurally similar compounds are obtained, which were also PPAR-γ agonists. 191 compounds were found in similarity search which are listed in table 8. These obtained compounds were dockedfurther against PPAR-γ receptor.

5.1.3- Molecular Docking

Molecular docking is a technique employed in computer aided drug design to predict preferred binding orientation of ligand with the receptor. Molecular docking includes binding of small molecules onto the active site of receptor and evaluating the binding affinity of their complex, is an integral part of *insilico* structure based drug discovery process. It is used to evaluate the stability of protein ligand complex formed in simulation period and their functional reliability inside living system, concluding the efficacy of drug. Molecular docking includes binding of small molecules onto the active site of receptor and evaluating the binding affinity of their complex, is an integral part of *in-silico*structure- based drug discovery process. It is used to get the predominant binding mode of protein ligand complex, formed during docking. Molecular docking was performed in AutoDock vina software (Ghose, A. K. et al, 1999). This software is a fastest open source program for virtual screening, molecular docking and drug discovery process offering high efficiency and increased accuracy. It relies upon simple scoring function and rapid gradient based optimization conformational search. The best part of Autodock vina's design philosophy is the user doesn't need to know its implementation details, obscure search parameters, calculation of grid maps, assigning atomic charges, advanced algebra and cluster results. The only required thing is molecular structure which is going to be docked and search space specification including binding site.

5.1.3.1- Protein, ligand and grid preparation-

The protein preparation was done in ADT tools. In the process of protein preparation, water molecules were deleted, polar hydrogens and Gasteiger charges were added and while preparing ligand, root was detected, torsion angles were set and aromaticity criterion was set,

both protein and ligand were saved as pdbqt files. The pdbqt files require all ligands (PPAR-γ agonists) and protein receptor (3V9T). The grid box was formed around co-crystalized ligand of the PDB 3V9T and grid center (dimensions: x=11.239, y=48.8, z=61.278) with grid size (x=46, y=40, z=40) as used for docking calculation in vina tool.

5.1.3.2- Docking interaction visualization-

The screened molecules obtained from PubChem database (191 compounds) were docked against PPAR- γ receptor in AutoDock Vina. Those result values which were less than 1.0 Å in positional root- mean- square deviation (RMSD) were taken together and shown by the value having most favored free energy of binding. The pose which was having lowest binding energy was selected and aligned with co- crystalized ligand for further analysis. Lowest energy docking position of each compound is shown in table 9. Out of 191 compounds, 9 compounds having highest dock score were selected and their docking picture and interactions are shown in table 10. These compounds were tested for ADME (Adsorption Distribution Metabolism Excretion) studies using different softwares.

5.1.4- ADME Studies

Absorption, distribution, metabolism, excretion i.e. ADME studies have key role in drug discovery. A lead compound should have proper efficacy against particular therapeutic target, and should display proper ADME characteristics at specific therapeutic dose. ADME profiling helps in decreasing potential risk in clinical development, because it screens those drugs which mostly fail to develop due to safety and efficacy issues. So ADME studies helps to clarify that whether a particular compound is safe to proceed for pre-clinical or clinical phase or not. Most of the compounds expected to act as a drug fail due to poor ADME properties so prior ADME studies are required during *insilico*drug development process. Therefore, ADME studies were done using different softwares considering common parameters among results of different softwares, as parameters shown in resultsof different softwares were different. For this, 5 softwares were used namely swissADME (Daina, A. et al, 2017), vNN Webserver (Schyman, P. et al, 2017), admetSAR (Cheng, F. et al, 2012), DRULITO (Kar, S. et al, 2020) and ADMETlab (Xiong, G. et al, 2021). As all these 5 different softwares were giving results in different parameters, so 17 common parameters were selected to compare the resultsof these different softwares. Parameters selected were MW, H bond acceptor, H bond donor, Log P, Lipinski Violations, TPSA, Bioavailability score, Log S, Log D, Rotatable bonds, MR, Log

Kp, Synthetic accessibility, PAINS, BRENK, PPAR-γ binding, and p-glycoprotein inhibitor. Combined result of these parameters is shown in table 16, whereas table 17 is showing range of values of different parameters taken in ADMET analysis for a good drug.

5.1.5- Molecular Dynamics

The MDS studies were performed by using Desmond 2022.1 to investigate about the changes in the protein–ligand complex conformation with solvent system. The process of MDS is done in multiple stages; (1): Formation of Setup (loading of data, solvation i.e. ions and water is added), (2): Energy minimization of protein, (3): Solvent is equilibrated around the protein, with both NVT and NPT ensembles, (4): then production simulation is done, this produces trajectory, which explains atomic motion of system, easy to be viewed and analyzed using other software. The OPLS forcefield was utilized for the MDS of docked complex (ligand_ PPAR-γ). Inside an orthorhombic cubic box, the position of complex was kept at center andbuffers along with TIP3P water molecules were filledat a 10Å distance, between box edge & protein atom for simulation. The boundary condition box volume was also computed for every complex type along with counter ions including Na^+& Cl^- which were addedto randomly neutralize the system. The X-ray ligand (cercosporamide derivative) was selected as the reference compound for comparison of simulation results of 3 compounds. Exploiting Desmond protocol, built solvated system was minimized, utilizing OPLS-2005 forcefield parameters followed by its relaxation.

Berendsen NVT ensemble was utilized and the simulation system was kept at the 10 K temperature for restraining of heavy atoms present on the solute. However, the MDS was done at 300K temperature, 1 atm pressure and 1ps thermostat relaxation time under isothermal isobaric ensemble (NPT). For MDS the Martyne–Tobias—Klein barostat (Martyna, 1994) and the Nose–Hoover thermostat (Evans & Holian, 1985) approaches were used for maintaining the temperature and pressure at 300 K and 1.01 atm, respectively. The progress of the simulation was recorded after every 200 ps. The NPT ensemble was formed which was followed by the simulation process, performed for 200 ns production. Afterwards, the trajectories were investigated by gathering the frames and examined by exploiting the simulation interaction diagram, generated by Desmond that further helped indetermining fluctuations.

5.2- <u>In Vitro study</u>:

All the equipments used were already calibrated as per their procedure.

5.2.1- DPPH Free Radical Scavenging Assay:

Antioxidant activity is essential aspect to deal with several diseases like cancer, diabetes, ischemia, heart disease etc. Antioxidants remove free radicals or reactive oxygen species generated by several metabolic activities. DPPH (1, 1 diphenyl-2-picrylhydrazyl) free radical scavenging activity was measured utilizing method explained by (Sharma et al., 2009). Standardization of DPPH assay was done by forming fixed DPPH solution, 0.1 mM in methanol or ethanol. Solution was freshly prepared or stored in the dark at 4°C (stable for a short period). Wavelength: Absorbance is measured at 517 nm (peak absorbance of DPPH). Incubation time: Standardized (often 30 minutes at room temperature in the dark). Solvent consistency was maintained by using high-purity methanol or ethanol, avoided water to prevent DPPH instability. Control and blank: Blank = solvent + DPPH (no antioxidant). Control = solvent only (baseline correction). Calibration was done by creating a reference curve using known antioxidant ascorbic acid. Then, % inhibition is measured using: = = $[(A_0 – A_1)/A_0]$ x100 formula then standard curve is plotted to calculate IC_{50} of unknown samples. Then, validation, which ensures reliability and reproducibility of assay, is done by using triplicate runs, linear dose-response relationship for standard is ensured, calculated antioxidant values were compared with standard. Validation also done based on standard deviation of blank and slope of calibration curve.

5.2.1.1- Principle:

This assay is used to measure antioxidant potential of test compound. It is basically an electron transfer method in which odd electron of nitrogen atom present in DPPH molecule gets reduced by getting hydrogen atom from antioxidant (test compound) to corresponding hydrazine. DPPH is a violet colored, stable free radical which after reduction by antioxidant converts into colourless solution. Stadardization

5.2.1.2- Reagents:

- DPPH
- Ethanol
- Ascorbic acid

5.2.1.3- Procedure-

- Firstly, 2.36 mg of DPPH was mixed in 50 ml of 100% ethanol to make finally 0.11mM DPPH solution.
- Then 0.1 ml of sample solution of compound silymarin and hesperidin, prepared by dissolving 5 different concentrations (10, 20, 40, 80, 160 µg/ml) in ethanol, was added to 3.9 ml of DPPH. These various concentrations were prepared by dilution method.
- In blank, ethanol was used instead of sample, whereas ascorbic acid was used as control.
- Then the whole mixture was shaken vigorously and allowed to rest at room temperature for 30 min.
- Then absorbance was taken using spectrophotometer at 517 nm.
- The % DPPH scavenging activity was calculated using below equation:

% DPPH scavenging activity or inhibition = $[(A_0 – A_1)/A_0]$ x100

Where A_0 was the absorbanceof control and A_1 was the absorbance in presence of test compound.

5.2.2- Cell Viability Assay-

Cell viability assay or MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was performed by the method described by Kumar et al, 2018. This method is used in studies involving cell lines to assess the effect of test compound on viability of cells used. We used MDA-MB-231 cell line, as it is a human breast cancer cell line, a widely used model in research for TNBC, known for its aggressive and metastatic nature. Standardization of MTT assay was done by using authenticated cell lines with low passage number, same density of cells across all wells, solvent DMSO, MTT 0.5 mg/mL, for 2-4 hours incubation time, maintaining 37°C and 5% CO_2, absorbance at 570 nm. Then, calibration is done to ensure linear relationship between cell number and absorbance. For this, standard curve cell density vs OD plot is made by seeding cells in increasing density and performing MTT assay. % viability is calculated. Validation confirm linear correlation between cell number and absorbance, ensure that only metabolically active cells reduce MTT.

5.2.2.1- Principle-

MTT assay is a colorimetric assay used basically to evaluate cell viability, cell cytotoxicity and cytostatic activity after treating cells with test compound. It is based on the notion that only living (viable) cells have active NADPH dependent oxidoreductase enzyme present in mitochondria, which can reduce MTT (yellow colored) to insoluble formazan (purple colored) crystals. These crystals are solubilized further in DMSO (Dimethyl sulfoxide) and viable cells

are quantified in colored solution by measuring absorbance in plate reader at 570 nm. Darker solution implies more metabolically active cells.

5.2.2.2- Reagents-

- MTT
- PBS (Phosphate buffer saline)
- DMSO (Dimethyl sulfoxide)

5.2.2.3- Procedure of estimation of cell viability-

- Firstly, cells were added at a concentration of 1 x 108 cells per well in 96 well plate and kept in a CO2 incubator at 37 °C and allowed to adhere for 24 hrs.
- Then after 24 hrs. Culture media was replaced by a fresh one.
- Then cells were treated with different concentrations of test compounds and kept at 37 °C in CO2 incubator for 24 hrs.
- After this again the culture media was replaced with fresh medium.
- Working solution of MTT made by dissolving 5mg in 10ml PBS was added in each well of 96 well plate and kept in CO_2 incubator at 37 °C for 4 hrs.
- MTT was then removed along with culture media and formed formazan crystals were solubilized by adding DMSO per well and incubating in CO_2 incubator at 37 °C for 30 minutes.
- Then finally absorbance was taken in ELISA (Enzyme linked immuno sorbent assay) plate reader set at 570 nm.
- Absorbance measured was directly proportionate with the % cell viability.

5.2.3- Cell lysis and preparation of cell lysate for protein array (6-well format)

Cells were lysed to obtain protein array in cell lysate. Phosphatase inhibitors and protease inhibitor were added to lysis buffer to prevent modification and degradation of extracted proteins and to obtain good protein yield in cell lysate. Lysis buffer was made by combining Tris, NaCl and EDTA (Ethylenediamine tetraacetic acid) in defined proportion. According to experimental design, cells were treated in 6 well plates and then washed with ice cold PBS. Then lysis buffer was added in 6 well plates and the cells were scraped off with the help of cell scraper and then obtained cell lysate was collected in micro centrifuge. Thus obtained cell lysate was then centrifuged in micro centrifuge tube for 7 minutes at 17000 rpm. After centrifugation, supernatant was collected and pellet was discarded.

5.2.3.1- Protein estimation-

Evaluation of protein concentration was done by standard method described by (Lowry et al, 1951), and a standard curve was made using bovine serum albumin (BSA). Stadardization ensures consistent performance of the reagents and procedures. It is done by preparing fresh reagents, control of conditions like maintain pH, temperature and incubation time, use standard protein bovine serum albumin (BSA). Blank is run to correct for background absorbance. Calibration relates absorbance readings to known protein concentrations via standard curve. Then validation confirms standard curve is linear. Repeat assay multiple time for precision.

5.2.3.1.1- Principle-

Lowry's method is used to assess the protein content of sample solution. In this method, firstly under alkaline conditions copper ions reacts with peptide bonds, which leads to oxidation of aromatic amino acid residues mainly tyrosine and tryptophan. In this process, Cu^{+}ions are produced which further reacts with Folin-Ciocalteu reagent. Folin- Ciocalteu reagent gets reduced and aromatic amino acids get oxidized in the process. Finally, intense blue heteropolymolybdenum blue is formed and its concentration is measured by taking absorbanceat 670nm.This way total protein concentration is deduced from the total tyrosine and tryptophan which reduced Folin-Ciocalteureagent i.e. colour produced.

5.2.3.1.2- Reagents-

- Reagent A = 2 % sodium carbonate in 0.1N NaOH
- Reagent B=1 % CuSO4 in Double distilled water (DDW).
- Reagent C = 2 % Sodium Potassium Tartarate in DDW.
- Lowry's reagent (Reagent D) = Prepared freshly by mixing reagents A+B+C in ratio 49:0.5:0.5(v/v)
- Diluted Folin- Ciocalteau's reagent with DDW in ratio 1:1(v/v)
- Stock protein standard: 10 mg of bovine serum albumin was dissolved in 10 ml of DDW.
- Working Standard: 1 ml of stock solution was diluted in 9 ml of DDW.

5.2.3.1.3- Procedure-

Table 5.1: Sample preparation in Lowry's method of protein estimation

Reagents	Blank(ml)	Standard(ml)	Test(ml)
DDW	1	0.9	0.9
Standard	-	0.1	-
Test Sample	-	-	0.1
Lowry's Reagent	5 ml to each	5 ml to each	5 ml to each

↓

Incubation done for 5-10 mins. at37^{o}C

↓

Then add fresh prepared 0.5ml Folin-Ciocalteau reagent in every tube

↓

Thenincubated again for30mins at37^{o}C

↓

Then absorbance was taken at670

5.2.3.2- Glutathione-s-transferase (GST) estimation-

The activity of Glutathione-s-transferase was estimated in cell lysate by method of Habig et al, 1974. Study ensures all assay components and conditions are optimized and consistent. Reagents are properly made and optimum conditions are controlled. Blank is used to zer the spectrophotometer. Then, Calibration is done by calculating specific activity of enzyme to draw linearity plot between absorbance vs time. Then validation confirms that absorbance increases linearly with time and enzyme concentration. Checks for precision, specificity and sensitivity.

5.2.3.2.1- Principle-

Glutathione-s-transferase is an enzyme which catalyzes the reaction that forms a conjugate between 1- chloro-2-4- dinitrobenzene (CDNB) and reduced glutathione (GSH). Thus formed conjugate is called as S-(2, 4-dinitrobenzene) Glutathionne, which absorbs at 340 nm.

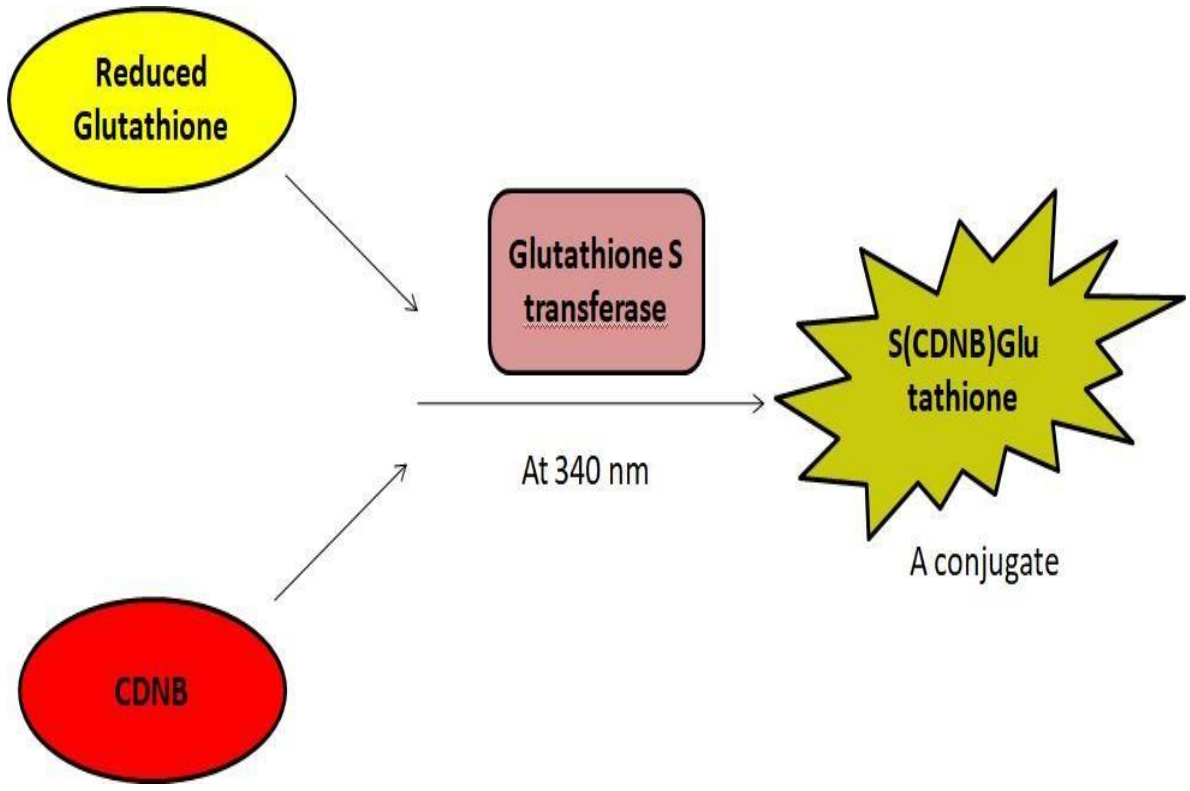

Figure 5.1: Showing principle of Glutathione-s-transferase activity

5.2.3.2.2- Reagents-

- 100mM Potassium phosphate buffer, pH=6.5
- 1mM Reduced glutathione (GSH), pH=6.5
- 15.3mg of GSH dissolved in 5ml of DDW
- 1mM CDNB
- 61mg of CDNB dissolved in 5ml of distilled alchohol

5.2.3.2.3- Procedure-

Table 5.2: Preparation of samples in Glutathione-S-transferase assay

	Blank (ml)	**Test (ml)**
Buffer	**1.0**	**1.0**
DDW	**1.65**	**1.55**
Reduced GSH solution	**0.3**	**0.3**
Sample	**------**	**0.1**
CDNB	**0.05**	**0.05**

The increase in absorbance observed as more and more glutathione conjugate forms, was recorded at 340 nm. using UV- VIS spectrophotometer for 5 mins. at intervals of 60 sec.

5.2.3.2.4- Calculations-

The activity of enzyme Glutathione-s-transferase was expressed as µmol.GSH conjugate formed/min/mgprotein.

5.2.3.3- Estimation of lipid peroxidation-

Estimation of lipid peroxidation was done by methodology given by Beuge and Aust (1978). Standardization ensures reagents and conditions are consistent for accurate detection of lipid peroxidation. Calibration links absorbance values to known MDA concentrations using a

standard curve**.** Validation confirms the assay is accurate, sensitive, and specific under various conditions.

5.2.3.3.1- Principle-

Cellular metabolism produces free radicals which can oxidize lipids especially polyunsaturated fatty acids (PUFA). Mainly OH and O_2 free radicals causes peroxidation of PUFA and leads to production of end product malondialdehyde (MDA), which along with thiobarbituric acid (TBA) produces pink color with absorption maxima at 532 nm.

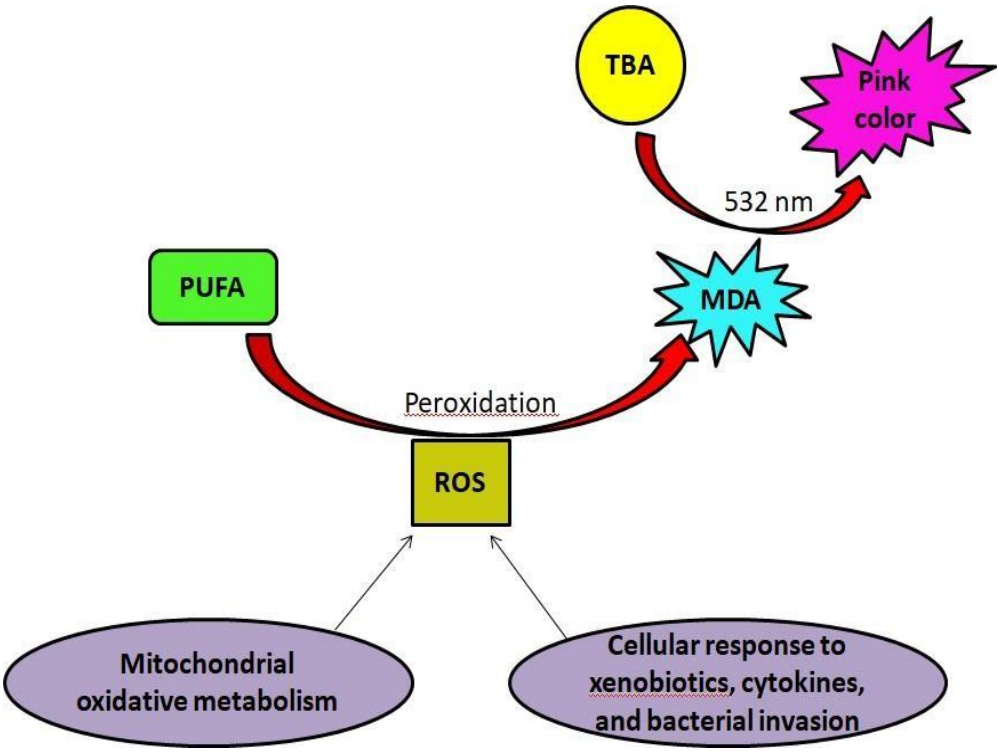

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Figure 5.2: Showing principle of lipid peroxidation

5.2.3.3.2- Reagents-

- 150mM Tris-HCl buffer, pH=7.1
- 1.5mM Ascorbic acid (prepared fresh)
- 1.0mM $FeSO_4$ (prepared fresh)
- 10% Trichloroacetic acid (TCA)
- 0.375% Thiobarbituric acid (TBA), pH=7.0

5.2.3.3.3- Procedure-

Table 5.3-Preparation of samples in lipid peroxidation assay

	Blank (ml)	**Sample (ml)**
Tris-HCl buffer	0.1	0.1
Sample	-------	0.1
$FeSo_4$	0.1	0.1
Ascorbic acid	0.1	0.1
DDW	0.7	0.6
Incubated at 37°C for 15 mins.	and stopped the reaction	by adding following
TCA	1.0	1.0
TBA	2.0	2.0

Then plugged the tubes and put in boiling water bath for 15 mins.

↓

Allowed to cool

↓

Then centrifuged at 3000 rpm for 10 mins.

↓

In supernatant absorbance was measured at 532 nm. and concentration of lipid peroxide was calculated.

5.2.3.3.4- Calculations-

The result of lipid peroxidation estimation was expressed as n moles MDA formed/mol.

5.2.3.4- Estimation of Catalase (CAT)-

Estimation of catalase activity was done in cell lysate by the method of Luck (1971). The catalase assay measures the activity of catalase enzyme, which decomposes hydrogen peroxide (H_2O_2) into water and oxygen. Proper standardization, calibration, and validation ensure the assay produces accurate, reproducible, and interpretable results. Standardization ensures the reagents and conditions are consistent and optimized. Calibration in catalase assay is slightly different. Catalase activity is not typically calibrated like a standard curve, but rather quantified via kinetic data. Validation validates that the method is accurate, reproducible, and suitable for its intended purpose.

5.2.3.4.1- Principle-

Catalase is an enzyme which catalyzes the reaction in which hydrogen peroxide breaks down into water and oxygen as follows-

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Breakdown of hydrogen peroxide by catalase causes reduction in its ultraviolet absorption with time. Released H_2O_2 has absorption maxima at 240 nm (in double beam spectrophotometer).

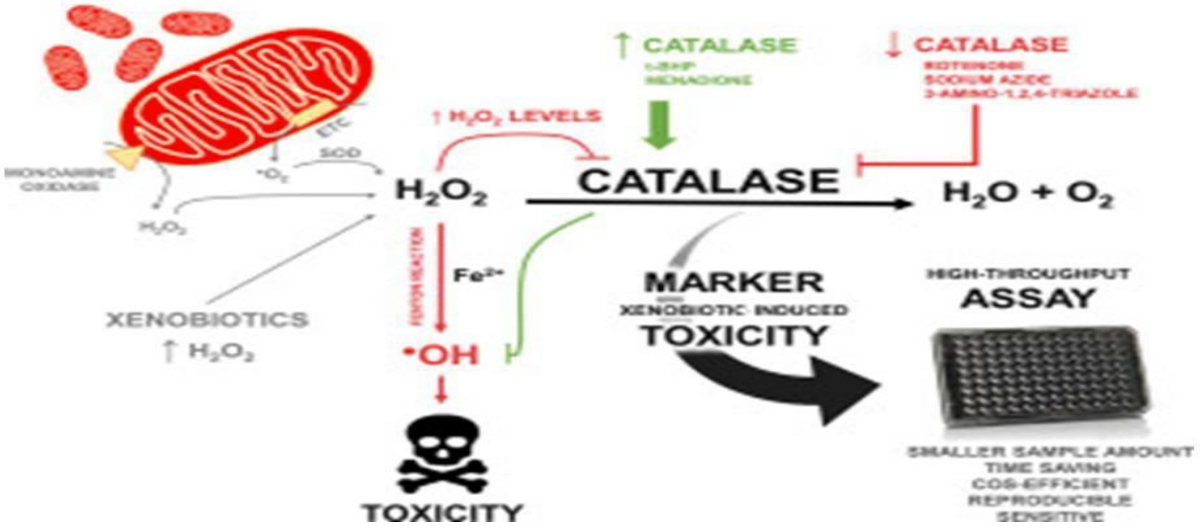


Figure 5.3: Showing principle of catalase activity

5.2.3.4.2- Reagents-

- 0.2M Phosphate buffer, pH=7
- Solution A made by adding 1.79gm of KH_2PO_4 in 200ml of DDW
- Solution B made by adding 3.52 gm of $Na_2HPO_4.2H_2O$ in 300 ml of DDW
- Then both solutions A&B were mixed in ratio 3:7 and the pH again adjusted to 7.0
- Working phosphate buffer was made by adding 160 µl of H_2O_2 to 100 ml of phosphate buffer

5.2.3.4.3- Procedure-

Table 5.4: Preparation of sample in catalase assay

Reagents	Blank	Test
Sample	25 µl	25 µl
Phosphate buffer containing H_2O_2	--------	3.0 ml
Phosphate buffer without H_2O_2	3.0 ml	-------

At 240 nm., reduction in optical density (O.D.) was measured for 2 minutes, after every 30 sec. interval.

5.2.3.4.4- Calculation-

Activity of enzyme catalase was calculated using 0.071 molar extinction coefficient of H_2O_2. Result of catalase estimation was expressed as µmole of H_2O_2 decomposed/min/mg protein.

5.2.3.5- Estimation of Superoxide dismutase (SOD)-

The estimation of enzyme superoxide dismutase activity was done by method explained by Kono (1978). The superoxide dismutase (SOD) assay quantifies the activity of SOD enzymes, which catalyze the dismutation of superoxide radicals ($O_2^{-}\bullet$) into hydrogen peroxide (H_2O_2) and oxygen (O_2). Proper standardization**,** calibration**,** and validation of the assay are essential for reliable and reproducible measurement. Standardization ensures consistent reagents, conditions, and assay protocol. For calibration, made and SOD activity is often reported in Units, not concentration. Validation confirms standard curve is the assay's reliability, accuracy, and reproducibility. Validation done by confirming % inhibition is linear with increasing SOD up to 50–80% inhibition and by determining specificity and sensitivity of SOD assay.

5.2.3.5.1- Principle-

Autooxidation of hydroxylamine hydrochloride generates superoxide radicals which mediates reduction of nitroblue tetrazolium (NBT) dye into blue colored formazon. Then the addition of superoxide dismutase stops the reduction of NBT, done by hydroxylamine hydrochloride. The extent of stoppage done by enzyme is considered as measure of its activity.

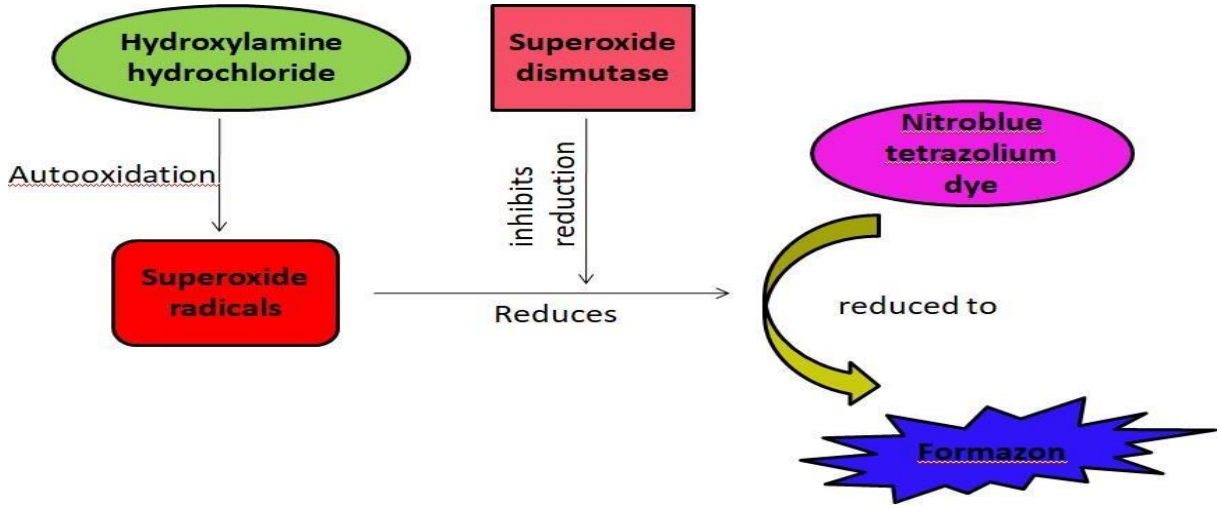


Figure 5.4: Showing principle of superoxide dismutase

5.2.3.5.2- Reagents-

- Sodium carbonate buffer (Na_2CO_3)- 50mM in 0.1mM EDTA, pH=10.8
- Triton-X-100 -0.6% (w/v) in DDW
- Nitroblue tetrazolium – 96 µM in 95% ethanol
- Hydroxylamine HCl- 20mM, pH=6.0

5.2.3.5.3- Procedure-

Table 5.5: Preparation of sample in SOD assay

Reagent	Blank (ml)	Test (ml)
Na_2CO_3	1.35	1.3
NBT	0.5	0.5
Hydroxylamie HCl	0.1	0.1
Triton –X-100	0.1	0.1
	Kept for 2 mins.	
Sample	-------	0.05

At 560 nm., development of blue colour was measured at every 30 sec. interval for 2 mins.

5.2.3.5.4- Calculation-

Enzyme concentration that provides half maximal stoppage of NBT reduction is defined as one unit of enzyme activity.

5.2.4- Western blotting-

Western blotting is a technique used in cell biology to separate and identify proteins from mixture of cellular proteins. For the data to be accurate, reproducible, and quantifiable**,** proper standardization**,** calibration, and validation are essential. Standardization ensures the western blot protocol is consistently followed with optimized reagents and conditions. Standardization ensures the western blot protocol is consistently followed with optimized reagents and conditions. Housekeeping protein actin was used as loading control to normalize the data. Molecular weight marker Bio-Rad Precision Plus Protein™ WesternC™ was used for detection of protein bands. Calibration ensures quantifiable and linear detection of protein bands. linear detection range of target protein and housekeeping control is determined. ImageJ software was used to quatify band intensity. Then standard curve is generated to get absolute quantification. Primary and secondary antibodies were calibrated to find the optimal dilution that gives strong, specific signal with minimal background. Validation confirms that the assay is specific, reproducible, and suitable for its purpose. Appropriate positive/negative controls were used and normalization and antibody validation was also done.

5.2.4.1- Principle-

It is based on the principle that proteins are firstly separated from protein mixture according to their molecular weight, then separated proteins are transferred from gel to membrane called immunoblot, then further blotted proteins are visualized by probing with primary and secondary antibodies.

1st Step:

5.2.4.2-Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS- PAGE)

This analytical technique was developed by Urich K. Laemmli, in this technique proteins are separated on the basis of their molecular weight.

5.2.4.3- Principle-

In SDS PAGE, proteins are separated solely on the basis of size or length of polypeptide chain, instead of structure or charge of proteins. During SDS-PAGEelectrophoresis, smaller protein

tends to migrate faster in gel in comparision to larger proteins, because

of decreased resistance from gel matrix. SDS provides negative charge to protein and thus mass to charge ratio is made same by it.

5.2.4.4- Reagents-

Stacking gel buffer, pH=6.8, (100ml)

- SDS -400mg
- Tris-6.05gm, pH =6.8

Separating gel buffer, pH= 8.8, (100ml)

- SDS -400mg
- Tris-18.2 gm, pH =8.8

Acrylamide 30x, (100ml)

- Bis-acrylamide -0.8gm
- Acrylamide -29.2gm

Staining buffer, (500ml)

- Acetic acid-50ml
- Methanol-250ml
- Brilliant blue-1.5g

Destaining buffer, (500ml)

- Acetic acid-50ml
- Methanol-250ml
- Water -250ml

1x Running buffer, (1 litre)

- Glycine 14.4 gm
- Tris 3 gm
- SDS 1 gm

4x Sample buffer, (10ml)

- SDS -0.8g
- 1M Tris -0.2g
- B-mercaptoethanol -4ml
- Glycerol -4ml
- APS -1ml (100mg in ml water)
- 10% Bromophenol blue -10µl

Table 5.6: Preparation of stacking gel and separating gel

Reagents	Stacking Gel (10 ml)	Separating gel (10 ml)
APS(Ammonium persulphate)	33.3µl	50 µl
30% Acrylamide	4.7 ml	1.3 ml
TEMED	10 µl	10 µl
Separating buffer	2.5 ml	2.5 ml
Water	2.83 ml	6.10 ml

Then polymerize for half an hour.

5.2.4.5- Protocol-

SDS-PAGE

- Extracted sample protein was suspended in sample buffer. Sample was then heatedfor 5 minutes at 95ºC.
- Then sample wascentrifuged for restoration of the volume of sample.
- Then stalking and separating buffer were prepared.
- 30 X acrylamide, stalking gel and separating gel were prepared.
- Then sample was loaded in the wells, along with standard protein.
- The gel was then run till stalking at the voltage of 70 V and after that gel was run for 1-2 hours at 140 V

5.2.4.6- Transfer setup-

- After completion of SDS-PAGE successfully, gel was removed then from the cassette of apparatus. and the well area and stalking of gel was then cut off.
- Then in process of transfer, tank was filled with transfer buffer.
- The gel was then transferred into transfer buffer, and shaken it for 10 minutes
- And the nitro cellulose membrane was also soaked in transfer buffer for atleast30 minutes.

2nd Step-

5.2.4.7- Immunoblotting-

5.2.4.7.1 Reagents Transfer buffer, 500ml

- Glycine -7.2 gm
- Tris -1.5gm
- Distilled water, (make up volume 500ml)

Tris-Buffered saline (TBS), pH= 7.2

- 2M tris-10ml
- 5M Nacl-30ml
- Milli que water -955 ml

TBST, pH= 7.2

- Added -0.5% Tween 20
- TBS-500m

2X blocking buffer

- TBS-500ml
- BSA (Bovine serum albumin)-400mg in 20ml TBS (for 1 blot)

Primary antibodies

- PPARγ Antibody (E-8): sc-7273
- Actin Antibody (2Q1055): sc-58673

Secondary antibody

- Goat anti-mouse IgG-HRP: sc-2005

5.2.4.7.2 Transfer assembly setup

- Firstly, the cassette was opened for making an assembly.
- Then sandwich was made, in which firstly foam pad was placed on one side of the cassettethen sheet of filter paper was placed, after which gel was placed on top of the filter paper.
- Then close to the gel, nitro ce000llulose membrane was kept, which was again followed by sheet of filterpaper, and then again second layer of foam pad was kept.
- Then the cassette was closed for transfer of proteins from gel to nitrocellulose membrane after formation of this sandwich.

5.2.4.7.3 Transfer method

- Sandwich assembly made inside the cassette was placed into transfer tank.
- Cathode (-) is kept at the side of the gelin cassette, whereas the membrane side faces the anode (+).
- After that inside the transfer tank, transfer buffer was added up to the mark.
- And then apparatus was run for 1 to 2 hours at 10-15 V till the completion of transfer of proteins from gel to nitrocellulose membrane.
- After completion of transfer of proteins, the sandwich was displaced from the cassette and then disassembled carefully with the help of forceps.
- After that transferred proteins were stained with amido black stain for verification of successful transfer.

5.2.4.7.4 Processing of immunoblot

- **Blocking** – Bovine serum albumin (BSA) was used as blocking buffer to reduce false positives. The nitrocellulose membrane was blocked by blocking buffer at room temperature (RT) for 2 hrs by using 2X BSA/0.1% tris buffer saline (TBS) duringcontinuous shaking.
- **Incubation with primary antibody**- Then the nitrocellulose membrane was further incubated along with primary antibody for whole night or 12 hrs. at 4ºC.
- **Washing-** Thus madeimmunoblot was then washed four times by 0.1% of tris buffer saline tween TBST. Firstly, washing was done two times for 5 minutes thenafter that again washing

was done two times for 10 min.

- **Incubation with secondary antibody-** Then the membrane was again incubated with secondary antibody at 37ºC for 1hr byshaking continuously.
- **Washing –** Then again the blot was washed by 0.1% of tris buffer saline tween TBST, four times. Firstly, washing was done two times for 5 minutes then after that again washing was done two times for 10 min.
- Then the final immuno blot was developed.

5.2.4.7.5-Detection and Quantification

The enhanced chemiluminescence (ECL) technology alongwith ECL hyperfilm were used for detection of proteins. Immunoblots were quantified by densitometric analysis with linear dynamic range exposure. For densitometric analysis, Image J software was used.

5.2.5- Transcription factor binding assay-

PPAR-γ transcription factor assay kit item no. 10006855 was used on MDA-MB 231 cells for transcription factor binding assay. It utilizes non-radioactive method for knowing specific transcription factor binding DNA activity in nuclear extract after cell lysis in 96 well ELISA. Standardization, calibration, and validation of PPAR gamma agonistic activity are essential for reliable and reproducible results in pharmacological and biochemical research. Standardization ensures uniformity across assays and experiments. Reference Standards: known PPARγ agonists (e.g., rosiglitazone**,** pioglitazone) as positive controls and vehicle controls (e.g., DMSO) as negative baselines are used. Media, serum type, cell passage number, incubation times, and reagent concentrations are used. Calibration correlates assay response with known agonist concentrations. Concentration-response curves for reference agonists is generated. Fit data to sigmoidal (logistic) curves using software (e.g., GraphPad Prism) to determine EC50 **(**half-maximal effective concentration) and Emax **(**maximum efficacy). Standard curve in every assay plate to correct for inter-plate variability. Normalize test compound responses to the reference agonist's response. plate reader is regularly calibrated using manufacturer's standards for luminescence, fluorescence, or absorbance. Validation assesses the reliability, accuracy, and robustness of the assay. Response is specific to PPAR-γ is confirmed. Verification is done that response increases proportionally with ligand concentration.

5.2.5.1- Principle-

This assay is based on the principle that PPAR-γ acts as transcrition factor and along with

its coactivators binds onto the PPRE sequence present on the DNA. So in this assay, PPRE (Peroxisome Proliferator response elements) sequence present on double strand DNA, was already immobilized on the bottom of 96 well plate. Then activated PPAR-γ present in cell lysate got bound specifically on PPRE sequence, immobilized on the wells, which further got detected by primary antibody directed towards PPAR-γ, which further got detected by secondary antibody. Then absorbance was taken at 450 nm.

5.2.5.2- Cell lysate preparation

- Cells were seeded firstly at a density of 1 x 108 cells per well in 96 well plates and allowed to adhere in a CO2 incubator for 24 hours at 37 °C.
- After cells got 70%-80% confluent, then cells were introduced into culture media which doesn't contain serum for 5 hrs.
- After this the cells were treated for one hour with silymarin, hesperidin, standard drug (doxorubicin) and positive control (pioglitazone) at concentration of 3µM, 6µM, 12µM, 24µM.
- Then the cells were lysed in lysis buffer (Tris HCL, NaCl and EDTA) 1 Molar sodium ortho vanadate, sodium fluoride, sodium butyrate, PMSF (Phenylmethylsulphonyl fluoride) and Tritn X.

5.2.5.3- Assay protocol

- All the reagents and cell lysate were added in 96 well plate according to instructions present on kit and then put on incubation for 1hour.
- 1x wash buffer was used for washing each well for five times.
- Then into the wells, 100µl of diluted primary antibody of PPAR-γ was added (except in blank well) and then kept on room temperature for 1 hour.
- Then each well was washed again by 1x washing buffer
- Further in next step, 100µl of diluted secondary antibody which is conjugated by HRP (Horse reddish peroxidase) was added in the wells and then again put on incubationat room temperature for one hour.
- Then wells were again washed by using 1x washing buffer
- Then 100µl of developing solution was added into the wells and then again 96 well plateswereput on incubationat room temperature for 30 minutes.
- Then after this 100µl of stop solution was added into the wells, which resulted into

colour change from blue to yellow.

- After this absorbance was taken after 5 min. of adding of stop solution at 450nm.

5.2.6- Real-Time PCR-

Real time PCR quatifies DNA concentration in real time. It is very sensitive and sophisticated technique. Triple negative breast cancer cell line MDA-MB 231 was used for treatment with silymarin and hesperidin along with standard drug doxorubicin. Standardization, calibration, and validation of Real-Time PCR (RT-PCR or qPCR) are crucial to ensure accurate, reproducible, and specific quantification of gene expression. Standardization ensures consistency and quality across qPCR runs. Total RNA was extracted from cell by TRIzol. The mRNA was then reverse transcribed into cDNA by the help of HiScript II One-Step RT-PCR Kit. Syber green quantitative Real time PCR (Polymerase chain reaction) (ChamQ master mix) was used according to mannufacturer's instructions and quantitative fluorescence analysis was done to assess the effect of these test compounds on the expression of antimetastatic genes like PPARG, BRCA1, SDC1 and genes responsible for apoptosis like p53, BCL2, BAX. B2M gene was used as a control house keeping gene. Relative quantification of gene expression was done by the method described by Livak et al, 2001. The sequences of primers (both forward and reverse primers) used for all the genes like PPARG, BRCA1, SDC1, p53, BCL2, BAX and B2M for annealing step of realtime PCR are given as follows:

PPARG-primers were: forward 5'-AGCCTGCGAAAGCCTTTTGGTG-3' and reverse 5'-GGCTTCACATTCAGCAAACCTGG-3'

BCL2 - forward 5'-TCGCCCTGTGGATGACTGA-3' and reverse 5'-CAGAGACAGCCAGGAGAAATCA-3'

BAX specific primers were: forward 5'-TGGCAGCTGACATGTTTTCTGAC-3' and reverse 5'-TCACCCAACCACCCTGGTCTT-3

House keeping

B2M primers were: **forward 5'-ACTGAATTCACCCCCACTGA-3' and reverse5'-AAGCAAGCAAGCAGAATTTGGA-3'** House keeping

BRCA1-primers were: forward 5'-CTGAAGACTGCTCAGGGCTATC-3' and reverse 5'-AGGGTAGCTGTTAGAAGGCTGG-3'

p53 (TP53) primers were: forward 5'-CCTCAGCATCTTATCCGAGTGG- 3' and reverse 5'-TGGATGGTGGTACAGTCAGAGC-3'

SDC1-primers were: forward 5'-TCCTGGACAGGAAAGAGGTGCT-3' and reverse 5'-TGTTTCGGCTCCTCCAAGGAGT-3'

Calibration ensures quantitative accuracy by relating Cq (Ct) values to template concentration. Standard curve is generated by plotting Cq vs log (copy no.). Housekeeping genes are used for normalization of data. Validation confirms the assay's specificity, reproducibility, accuracy, and dynamic range**.** Melt curve (SYBR) or probe signal (TaqMan) is used to verify single product by gel. Then, validated via standard curve (90–110%) with $R^2 > 0.98$. Primer-dimers or contamination is checked and confirm no genomic contamination is present.

5.2.6.1- Principle-

In this technique, amplification and quatification of DNA is done simultaneously by using fluorescent dye which intercalates between double stranded DNA and modified probes of DNA oligonucleotide, which fluoresce after hybridizing with complementary DNA.

5.2.6.2- Reagents-

Thermocycling was done in a total volume of 15 µl which included following things-

- 1 µl cDNA sample
- 0.5 µM Primer
- 4mM $MgCl_2$
- 1.5 µl Syber green quantitative Real time PCR (ChamQ master mix)
- Light cycler instrument and software version 3.5 (Roche applied science)

5.2.6.3- Procedure-

- Firstly, denaturation of sample cDNA was done for 10 min at 95ºC.
- Then, annealing of primer takes place at 55 ºC for 10 seconds.
- Further, extension took place for 18 seconds at 72 ºC.
- Like this, 45 cycles of denaturation, annealing and extension took place with PCR conditions as 95°C for 5 min (1×), followed by 95°C for 10 sec, 63°C for 30 sec (40×), 60°C for 10 sec (1×), and then 95°C for 10 sec (1×).
- During completion of DNA amplification in PCR cycles, melting curve analysis was done by measuring fluorescence continuously.
- Change in gene expression was assessed by calculating fold change for every gene in consideration.

5.3 - In-vivo Assays-

In-vivo assays were performed in fertilized eggs to check the anticancer potential of more potent compound Hesperidin, which was found better than Silymarin in *invitro* assays. In *invivo* assays, CAM angiogenesis which imparts information about formation of blood vessels was performed and cell invasion assay was also performed to check the potential of Hesperidin to inhibit metastasis of cancer cells in fertilized egg.

5.3.1- CAM (chorioallantoic membrane) Angiogenesis assay-

It is revealed from the literature that CAM assay had been used from earlier times as animal model for growing tumor grafts. Murphy and Rous (1912) had used CAM assay for transplanting chicken sarcoma. In a study by Dagg et al. (1956) first time growth of human cancer cells were assessed by CAM assay, and in 1980 in a reported study metastasis was studied by CAM assay (Ossowski et al, 1980). Recently, CAM assay gained popularity as a 3D animal model for various types of malignancies. CAM contains rich network of blood vessels, thus extensively used to assess angiogenesis and to screen novel antiangiogenic compounds (Tufan et al, 2005). Further CAM assay had also been used to study migration, growth and invasion (Cimpean et al., 2008; Ribatti, 2016). It is also used as model for preclinical study to screen and test anticancer compounds (Kue et al., 2015). In several studies CAM assay had also been used recently as a platform for patient- derived xenografts (PDXs) using surgical specimens of tumor in preclinical research (DeBord etal., 2018). It is a simple and versatile animal model for angiogenesis, tumor formation and metastasis. It is realized by researchers worldwide that CAM assay is a powerful tool for precision cancer therapy. Its usage is even more important in boron neutron capture therapy to know new anticancer boron-10 reagents. Thus based on these evidences, we tested compound 1 (silymarin) and compound 4 (hesperidin), obtained from *insilico*studies at 2 different concentrations below IC50 (Inhibitory concentration 50) i.e. 6 μM and 12μM to test the efficacy of compound for antiangiogenesis activity. Ensuring standardization, calibration**,** and validation is essential to obtain reliable and reproducible data. Standardization refers to ensuring that all experimental steps are uniform and repeatable. Eggs should be of same reed and age, 7-10 days incubation at 37–38°C**,** 60–70% humidity. Standardization of egg rotation, windowing procedure, and timing of membrane exposure was done. Volume and concentration of test compounds was stadardized. Consistent delivery method was used. Positive controls**:** Known pro-angiogenic compounds

(Doxorubicin). Negative controls**:** Vehicle-only treatments (e.g., saline or DMSO). Same magnification, lighting, and imaging equipment. Time points for image capture (e.g., 48 or 72 hours post-treatment) is fixed. Calibration establishes quantitative baselines and comparisons. Dose-response curve with known angiogenesis inducers/inhibitors to calibrate the assay's sensitivity is formed. Image analysis software ImageJ was also calibrated by calibrating camera and light source for consistency across experiments. Scale bars are used for size calibration in microscopy images. Validation ensures the assay is specific, sensitive, accurate, and reproducible. Confirms that observed vascular changes are due to the test compound and not other variables. Multiple eggs per group ($n \geq 6–10$) are used to account for biological variability.

5.3.1.1-Principle-

CAM assay is done by implanting a membrane containing test compound onto the chorioallantoic membrane of fertilized chicken egg, by cutting a hole in egg shell. After that when the CAM gets fixed then number of blood vessels generated due to presence of test compound, is counted by counting the branch points of number of blood vessels.

5.3.1.2-Materials required-

- Fertilized chicken embryos
- 70% ethanol
- Filter-paper disc (6mm)
- Parafilm (packing film)
- Phosphate buffered saline (PBS)
- Incubator
- Forceps
- Analysis software - Angiogenesis Analyzer plugin by Gilles Carpentier for ImageJ 1.48v

5.3.1.3- Sample preparation-

20mg of test samples PC, C1 & C2 were dissolved in minimum volume of DMSO (0.1% finalvolume) and made upto 1mL in PBS to get a stock solution of 20mg/mL. This was further diluted in PBS to get a working solution (WS) of 0.5mg/mL.

NC - 12µL PBS per disc Samples PC, C1 & C2:

Test concentration 1 - 6µL per disc

Test concentration 2 - 12µL per disc

5.3.1.4- Procedure-

1. Firstly, fertilized chicken eggs were cleaned with distilled water and tissue, and then incubated in an incubator for 2 days at 37^{o} C and 60% humidity.

2. Then further, after the disinfection with 70% ethanol of the shell center outside the air sac, a hole highlighted with marker pen is buffed and drilled gently over the air sac with a nipper not, for breaking the shell, and thus the vascular zone becomes easy to be identified on the CAM.

3. Then to moisten the inner shell membrane adjacent to the CAM, two drops of PBS are then added so that the membrane is easy to be separated from CAM.

4. Then the membrane and the CAM separated unforcedly, after being clamped and raised by ophthalmic forceps, then the window on the membrane is sectioned for exposing the vascular zone.

5. Further, 6mm diameter filter-paper discs were loaded with indicated concentrations of test sample or left blank and then directly applied and adhere to the vascular zone.

6. Then, upon sealing the openings with sterile packing film, the eggs were further incubated for 24to 48h.

7. Then blood vessels were viewed, photographed and quantified by using software for counting the number of blood vessel branch points.

5.3.2- CAM cell invasion (metastasis) assay-

Along with being used for angiogenesis, CAM assay is also used to study cell invasion and metastasis (Deryugina, E. I et al, 2008, Tufan, A.C. et al 2005, Ossowski, L.et al, 1988, Zhai, Y. et al, 2007). CAM model has several benefits like efficient tumor cell grafting due to highly vascularized nature, simplicity, cost effectiveness, high reproducibility, CAM being closed system so experimental compounds stay longer in comparision to animal models which allows study of antimetastatic compounds available in less amounts (Cimpean, A.M. et al, 2008). CAM contains ectoderm (outer), mesoderm (middle) and endoderm (inner) near to allantoic sac. CAM also has extracellular matrix proteins (ECM) like laminin, fibronectin, integrin and collagen type I. Presence of these ECM proteins physiologically mimics the environment of cancer. CAM assay is anestablished model for cell invasion in cancer like prostate cancer,

osteosarcoma, leukemia and bowel cancer etc. Standardization, calibration, and validation of a cell invasion assay such as the Boyden chamber (Transwell) or 3D Matrigel invasion assays are critical to ensure reproducibility, reliability, and biological relevance of experimental data. Standardization ensure uniform assay conditions across different experiments and laboratories. Same passage number and culture conditions are maintained. Cell identity was validated and tested for mycoplasma contamination. Consistent ECM substrate (e.g., Matrigel) at a defined concentration and volume was used. Number of cells seeded per insert were fixed. Calibration of the technique was done to ensure the assay system is functioning correctly and quantitatively. Positive and negative cotrols were used and validation was done for linearity, accuracy, specificity and sensitivity of the result. Confirmation is done for assay response linearity with increasing cell number or invasion potential.

5.3.2.1- Principle-

In the cell invasion assay, cancer cells are seeded in the upper chamber of two compartment microplate. Then a chemoattraction factor is added into the bottom chamber. Then assessment of number of cells passing from upper chamber to bottom chamber through the membrane is done.

5.3.2.2- Materials-

- Fertilized chicken embryos
- 70% ethanol
- Parafilm (packing film)
- Phosphate buffered saline (PBS)
- Incubator
- Forceps
- 20 G needles, BD, Biosciences
- 2. 30 G needles, BD, Biosciences
- Cell line- MDA-MB-231 Human breast cancer cell line, NCCS Pune.

- Cell culture media – DMEM (Dulbecco's modified eagle medium) medium supplemented with 10% Foetal Bovine Serum (FBS), MP Biomedicals, Germany
- 1X Dulbecco's Phosphate Buffered Saline (DPBS), 0.25% Trypsin-EDTA solution, MTT reagent, were all purchased from MP Biomedicals, Germany
- Dimethyl Sulfoxide (DMSO), cell culture grade, Merck, Germany
- Cell culture treated T-25 flasks from Biolite, Thermo Fisher Scientific Inc., USA.
- Geltrex LDEV-Free reduced growth factor basement membrane matrix, Invitrogen, USA
- Microscope - XDFL series, Sunny Instruments, China
- Analysis Software – ImageJ (Fiji) software V1.53j

5.3.2.3- Sample preparation-

20mg of test samples PC & C1 were dissolved in minimum volume of DMSO (<0.1% final volume) and made upto 1mL in PBS to get a stock solution of 20mg/mL. This was further diluted inPBS to get a working solution (WS) of 0.5mg/mL.

- NC - 12µL PBS
- Sample PC – 6 µL
- Sample C1:
- Test concentration 1 - 6µL
- Test concentration 2 - 12µL

5.3.2.4- Procedure-

1. Firstly, the MDA-MB-231 cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and grown at 37°C and 5% CO2. The cells were passaged at regular intervals twice a week.
2. Fertilized chicken eggs were incubated with intermittent rotation (37.5°C, 65% humidity, 10 days). On day 10, eggs were put on a rack; and a hole was drilled in the air sac at the blunt end of the egg using a 30-gauge syringe. Further, another hole was made near the allantoic vein while using a lightsource to control the drilling and to avoid causing an injury to the CAM. After that, another hole was drilled with a 20-gauge syringe needle and a mild vacuum was applied to the air sac holeto drop the CAMfrom the shell.

Figure 5.5: Showing fertilized chicken eggs used for cell invasion assay

3. Further, a square window (~1 cm^2) was made close to the bifurcation of the allantoid vein. Then, 25 µl of the suspension of ~1 × 10^6 MDA-MB-231 cells in the serum-free medium mixed with 25 µL Geltrex LDEV-Free reduced growth factor basement membrane matrix (Invitrogen) was applied close to the allantoid vein bifurcation using a pipette while avoiding a directcontact with the CAM. After xenografting, the eggs were left standing upright for 5–10 min in order to allow the cells to settle, sealed with tape, and left to grow (4 days at 37.5°C) on a stationary incubator.

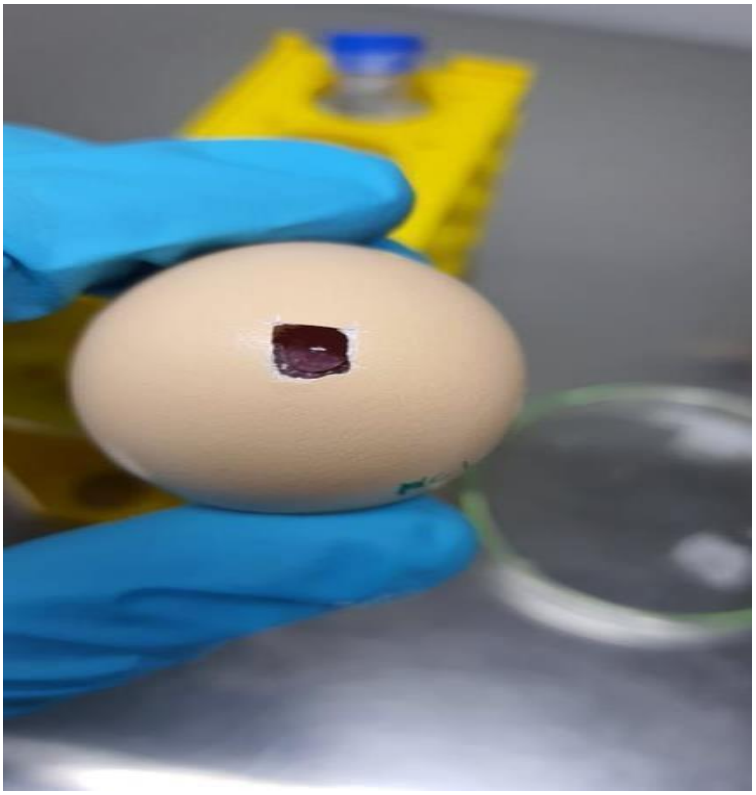


Figure 5.6: Showing windowing in egg

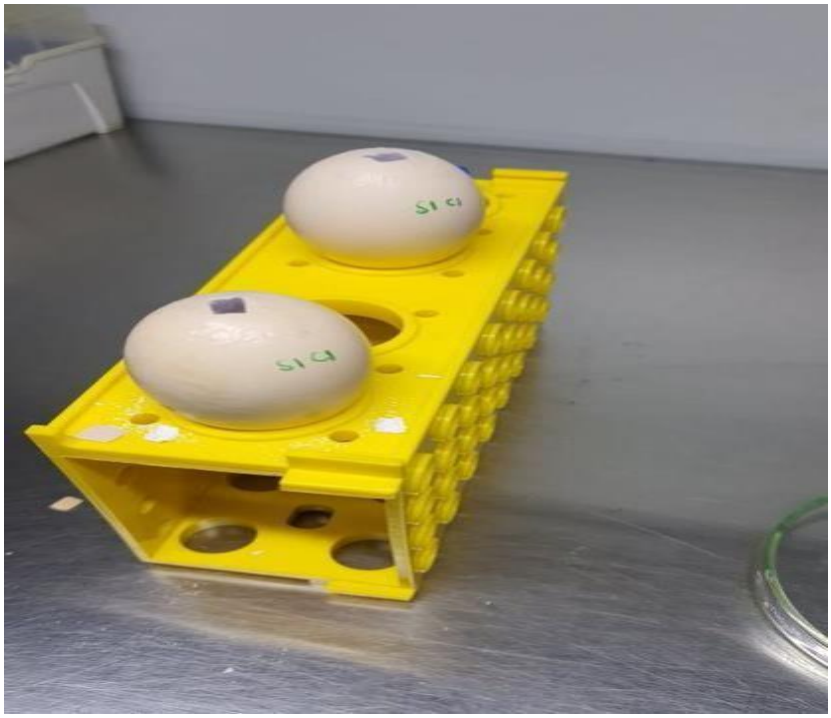


Figure 5.7: showing sealed windowed eggs

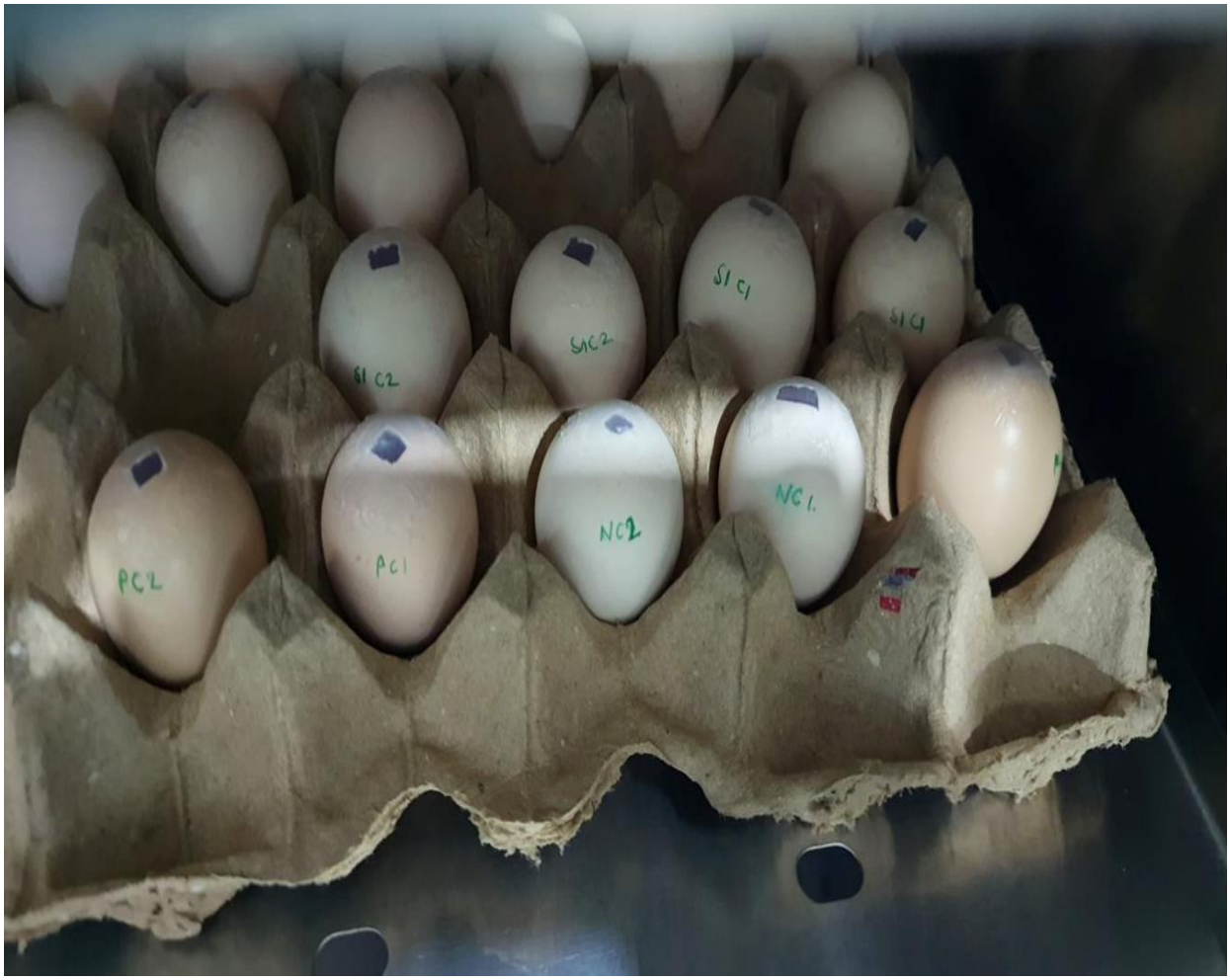


Figure 5.8: showing different sealed egg used in duplication

4. Then, required volume of test sample was added topically directly onto each microtumor on the upper CAM and the eggs were left without movement for 30 min to enable absorption to the microtumor and to avoid possible runoff from the microtumor sites. After that, the eggs were incubated for additional 48h. Parts of the CAM were extracted to carry out immunohistochemistry studies.

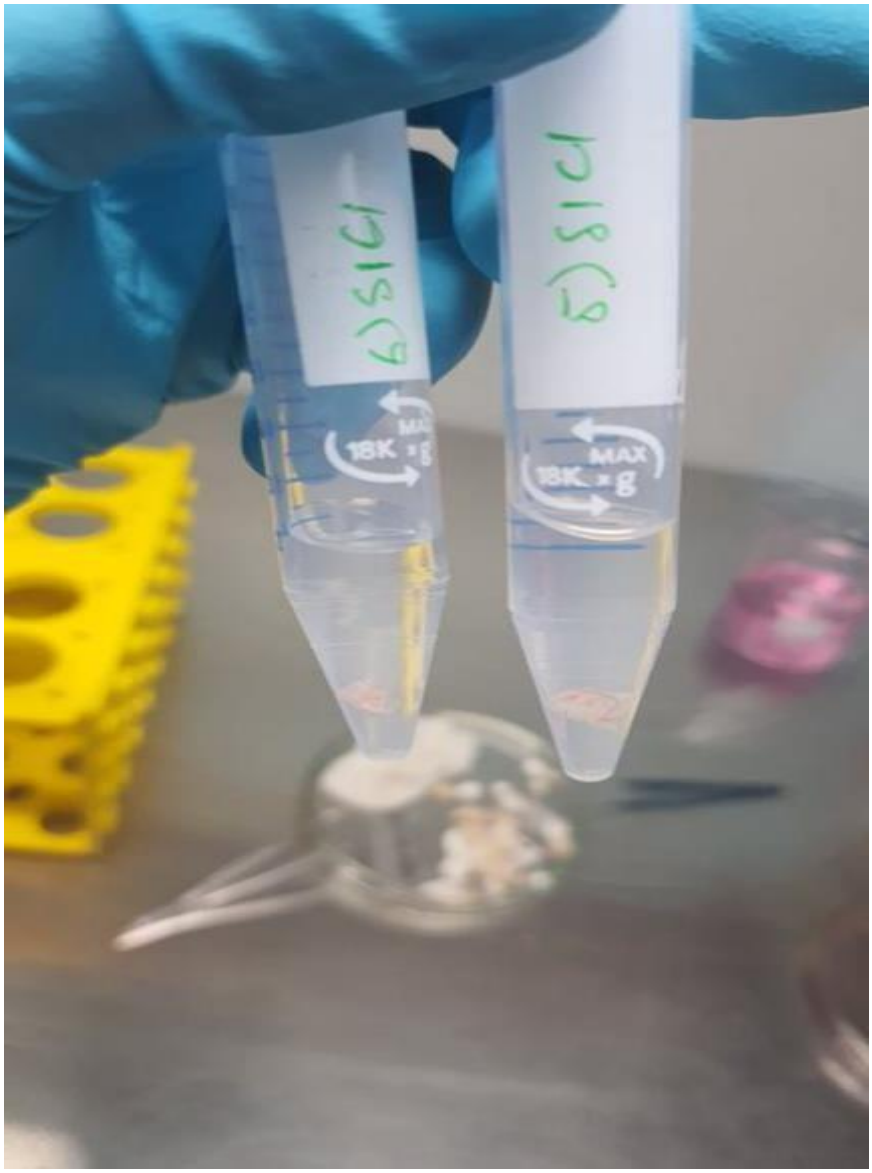


Figure 5.9: showing sample to be used in assay

5.3.2.5- Immunohistochemistry-

5. After extraction, tissues were embedded in paraffin and cut usingmicrotome; and 5-µm sections were placed on poly-L-lysinetreatedslides. Before staining, slides were deparaffinized, andtissues were rehydrated.

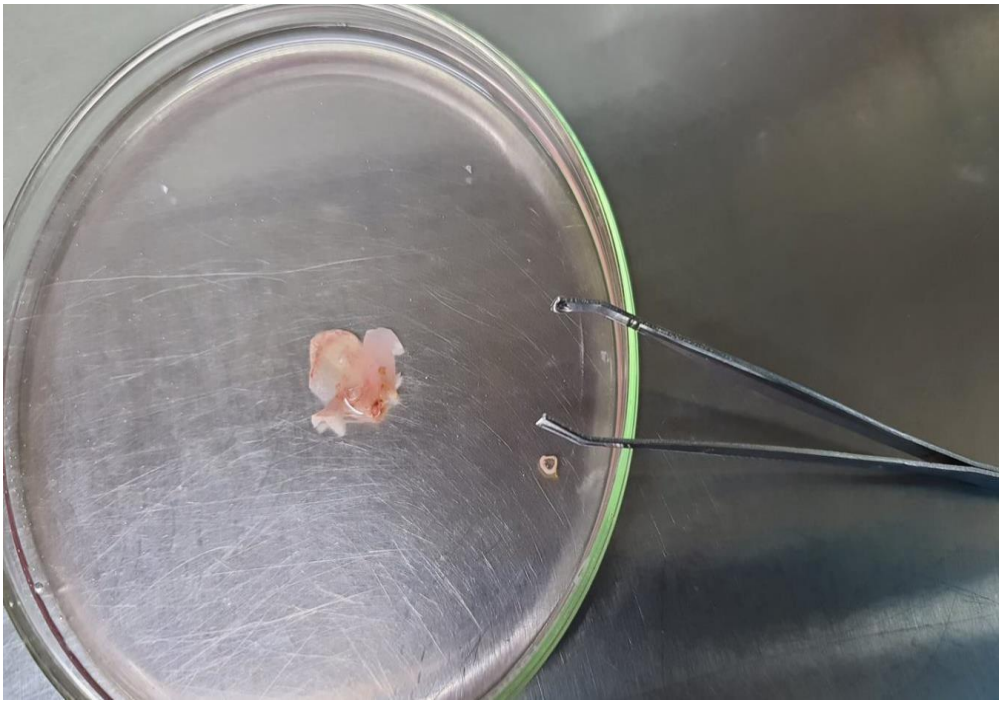


Figure: 5.10 showing sliced tissue

6. After that, slides were dried (1 h at 60°Cor overnight at 37°C). Then, permeabilization of cells was carriedout by incubation (10 min) of tissues with 0.1% IGEPAL in 1×Tris Buffered Saline (TBS). After that, the specimens werewashed (2×, 5 min with 1× TBS), and non-specific bindingswere blocked using a serum-free blocking agent, background punisher (BIOCARE Medical, USA), for 8 min.
7. Then, 1% bovine serum albumin (BSA) 0.05% IGEPAL in 1×TBS solution with a primary mouse anti-human Ki67 antibody (diluted 1:100) (Invitrogen USA) orwithout primary antibody (negative control) was incubatedovernight (4°C).
8. After incubation, specimens were washed in1× TBS and incubated with 3× hydrogen peroxide in 1× TBS for20 min to quench endogenous peroxidase.
9. After that, the slideswere washed again using 1× TBS and incubated (1 h) with asecondary anti- rabbit biotinylated antibody (Invitrogen USA) (dilution 1:400 in 1% BSA0.05% IGEPAL in 1× TBS). Then, slides were washed, asdescribed above, and tissues were incubated (30 min) withhorseradish peroxidase avidin D (dilution 1:500 in 1× TBS).
10. Finally, slides were washed again and incubated (5 min) with diaminobenzidine (Gibco, USA). In addition, tissues were stained with hematoxylin, dehydrated, cleared, and mounted with DPX mountant. Micrographs were captured using microscope.

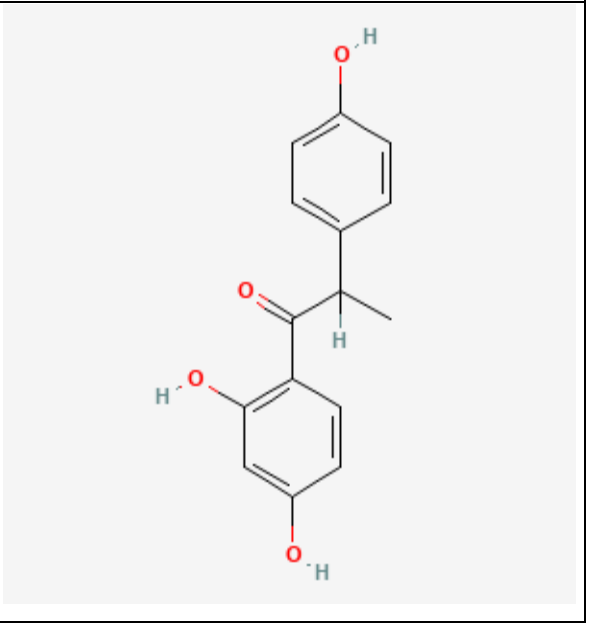
CHAPTER 6
RESULTS

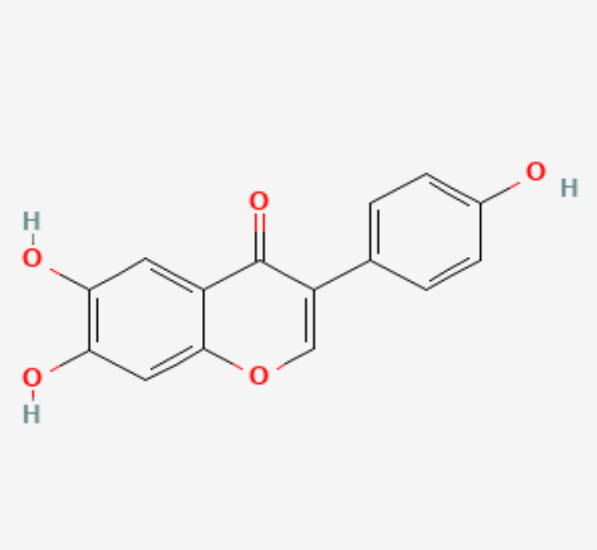
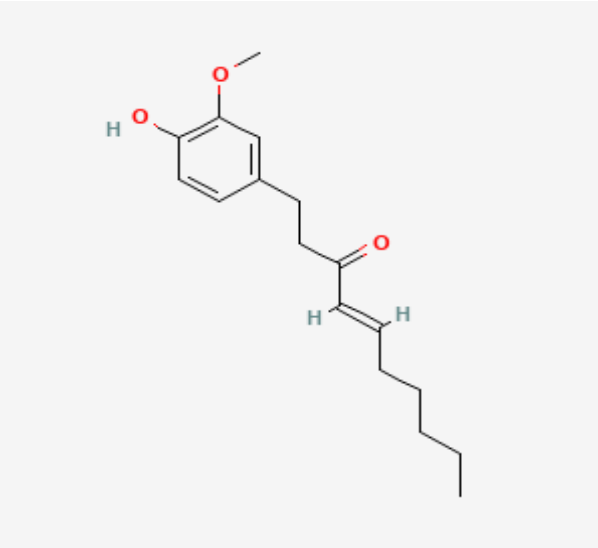
RESULTS

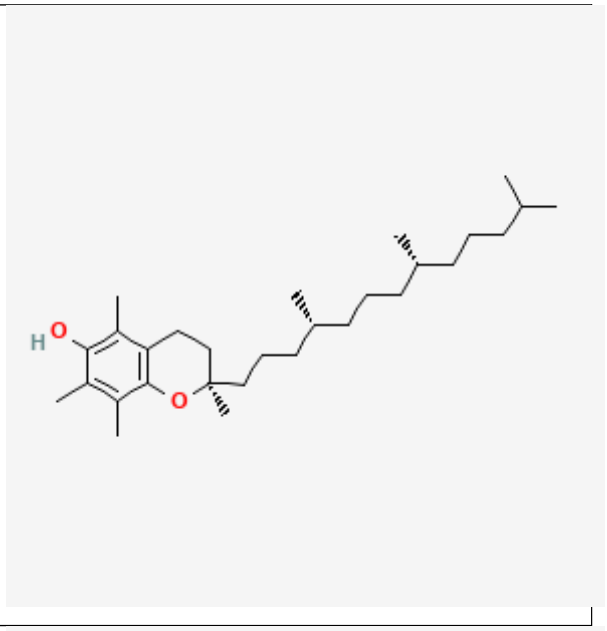
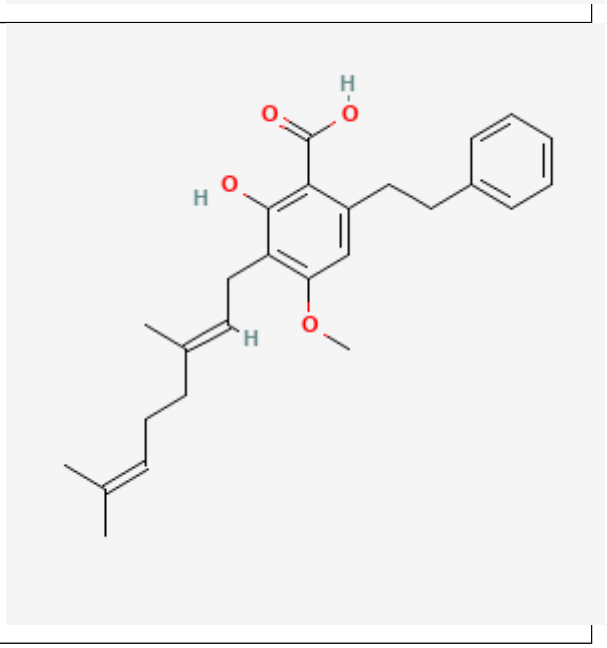
6.1- Data mining and Similarity Search

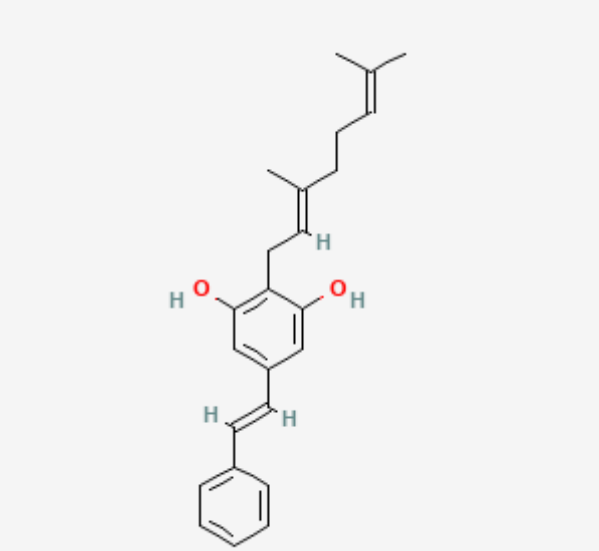
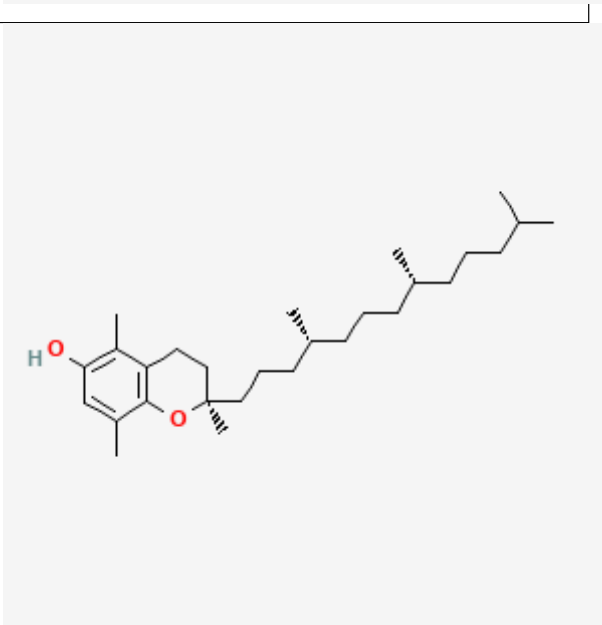
Similarity search is based on the notion that if two structures are similar then both the structures will share similar biological properties too (Martin et al., 2002). Thus, to explore more compounds having anticancer properties and PPAR-γ agonistic properties, it was important to know the structure of known compounds which were already established as PPAR-γ agonists having anticancer properties. As based on literature review it was found that PPAR-γ has ameliorating effect against TNBCand its agonists are found to activate it. So, in similarity search 47 reported natural PPAR-γ agonists (Wang et al, 2014) were used as query sequence. Their 2D structures were obtained from PubChem database, and are given in table 6.1. These reported natural PPAR-γ agonists, were used as query sequence to search PubChem database on the basis of fingerprint Tanimoto-based 2-dimensional similarity search with similarity index more than 80%. From this search, 191 compounds screened on the basis of similarity, are enlisted in table 6.2.

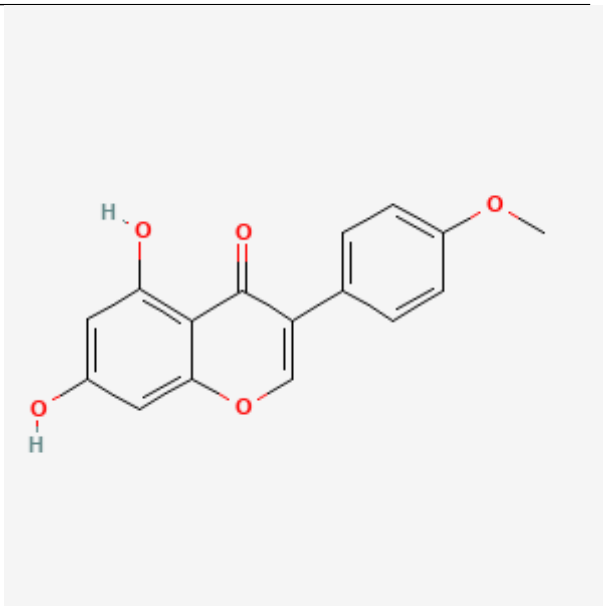
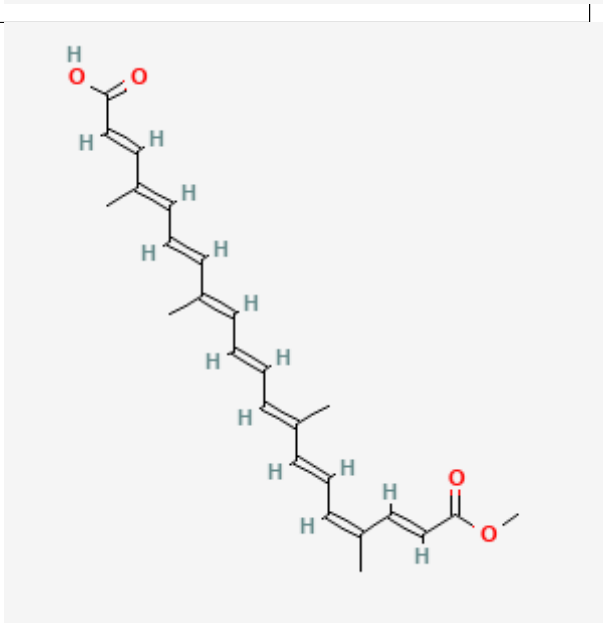
Table 6.1: Showing 2D structure of reported natural PPAR-γ agonists taken as query.

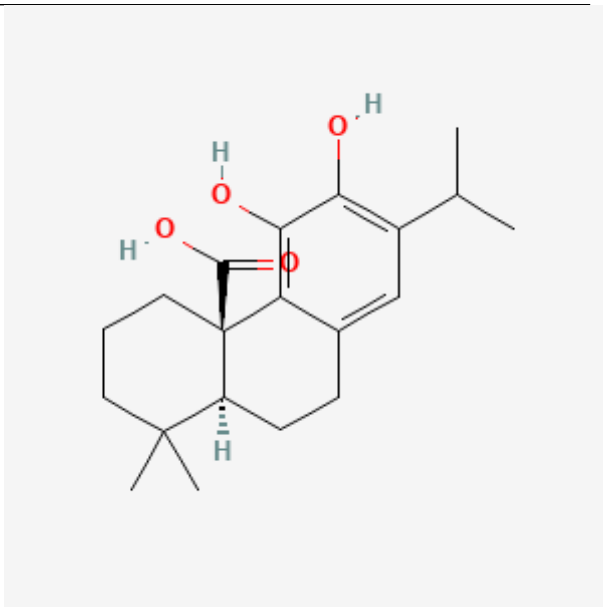
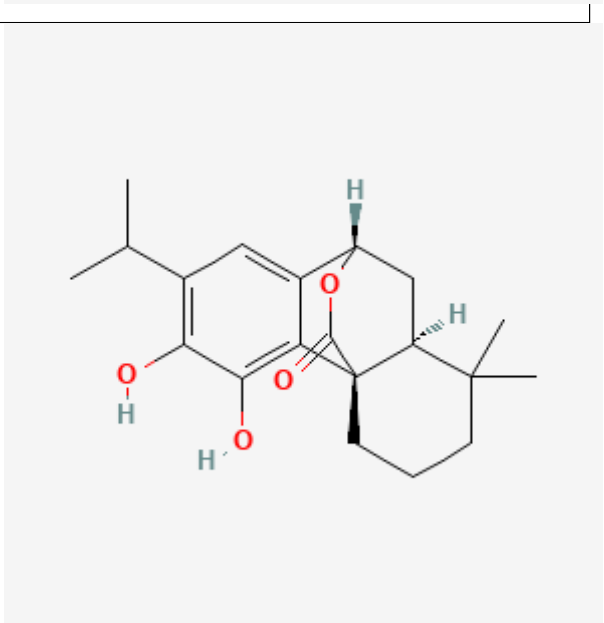
Natural ligands of PPAR γ	Structure
1. 6_hydroxy_o_desmethylangolensin	

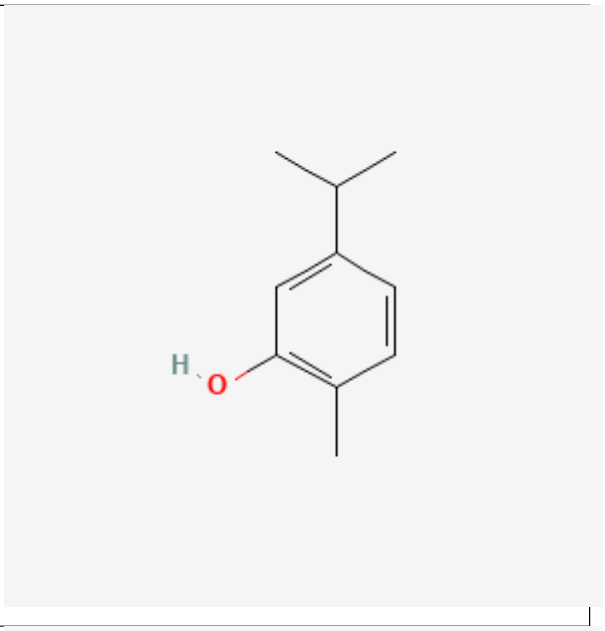
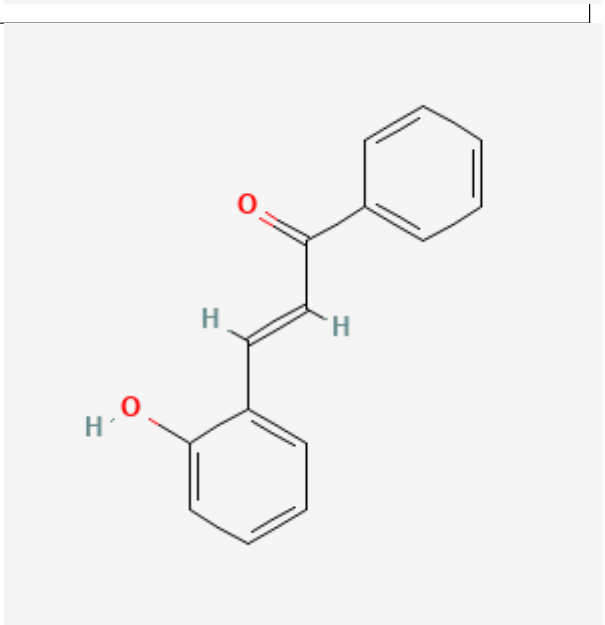
2. 6_hydroxydaidzein	
3. 6_Shogaol	

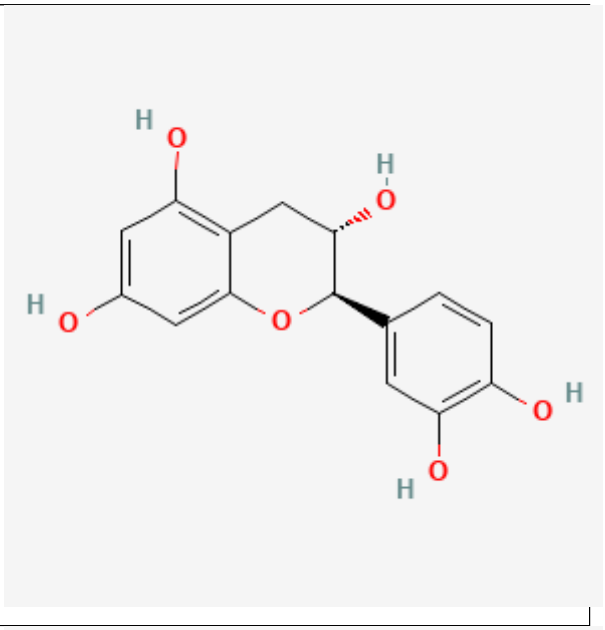
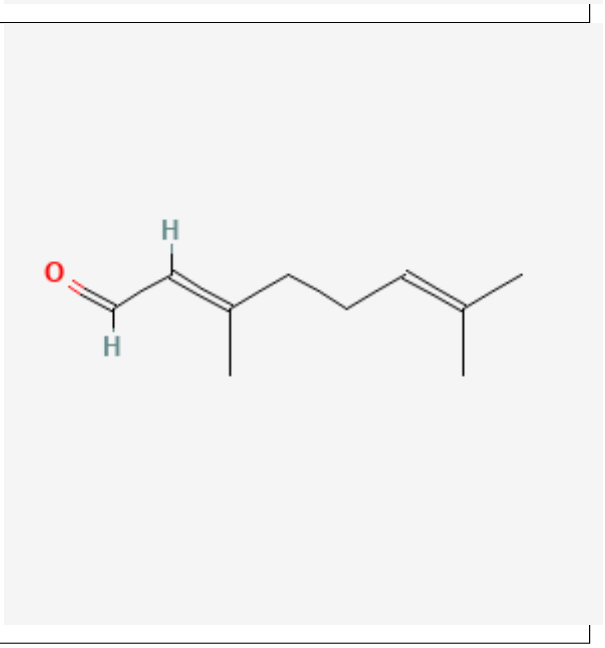
4. Alpha_tocopherol	
5. Amorfrutin_B	

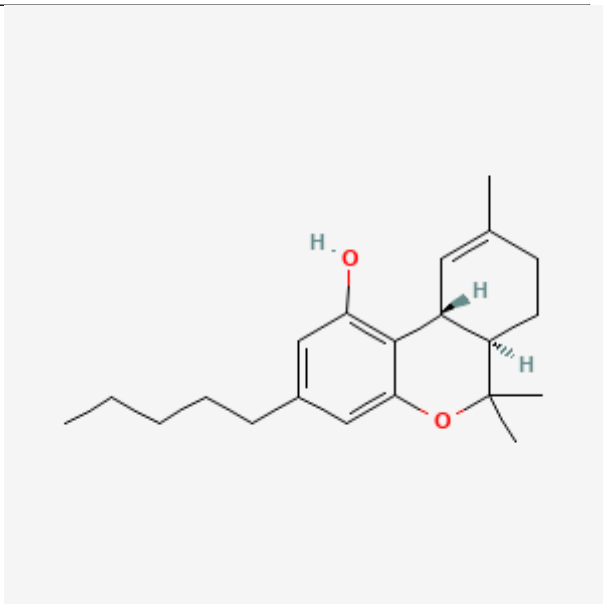
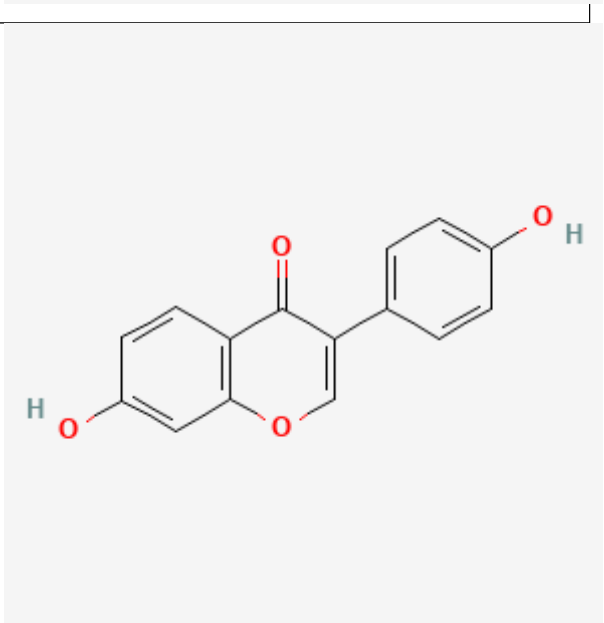
6. Amorphastilbol	
7. Beta_tocopherol	

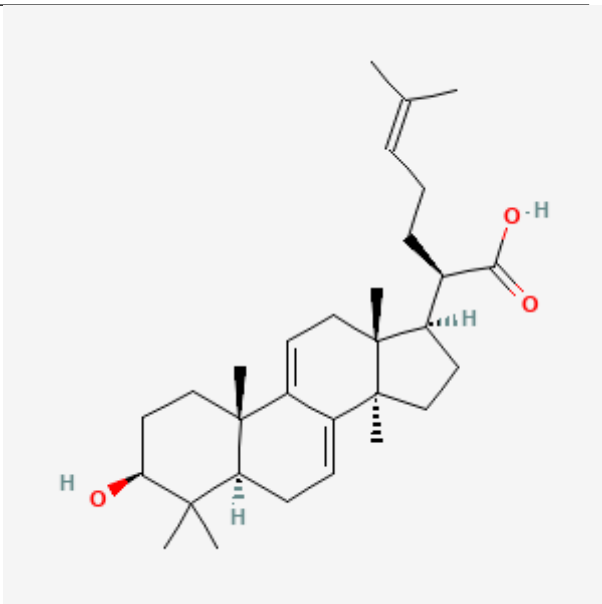
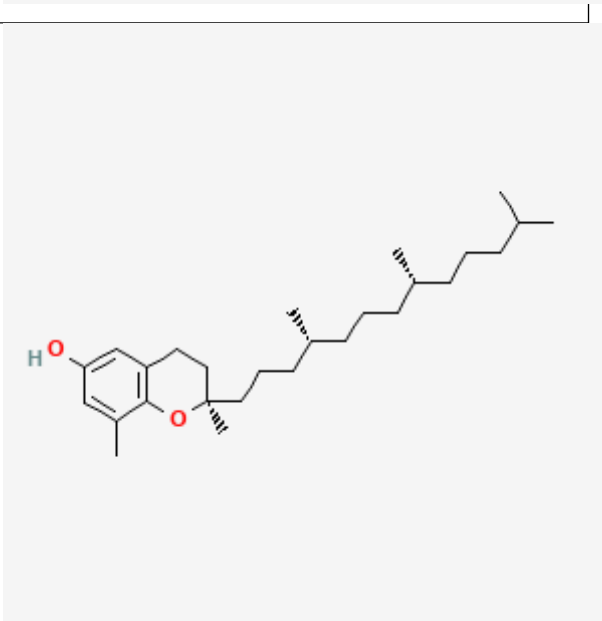
8. Biochanin_A	
9. Bixin	

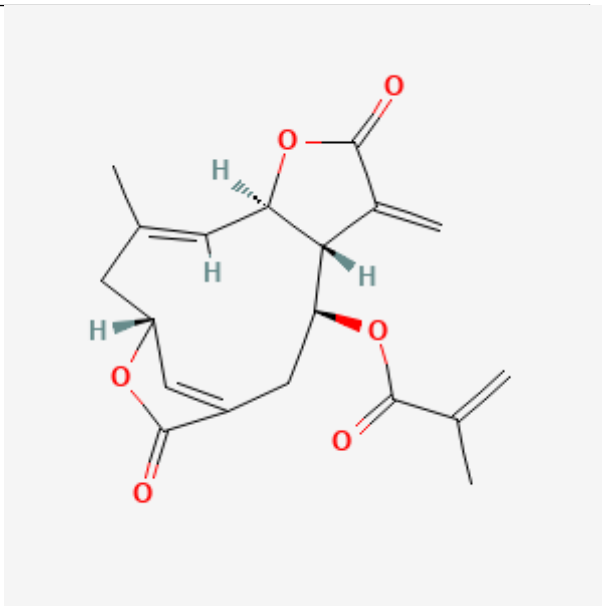
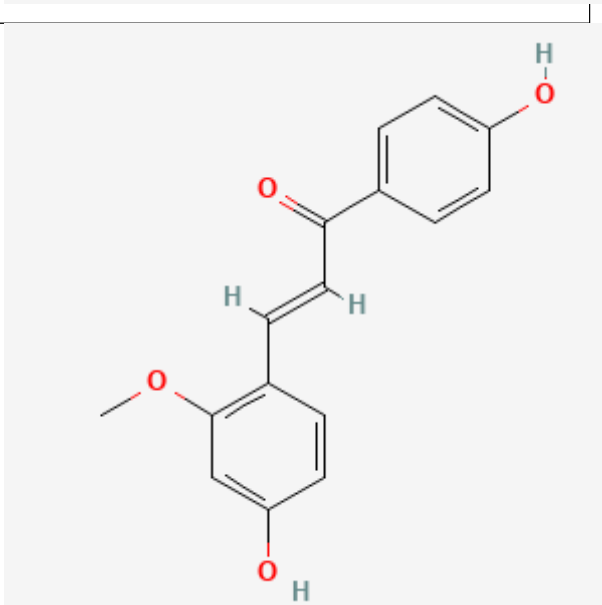
10. Carnosic_acid	
11. Carnosol	

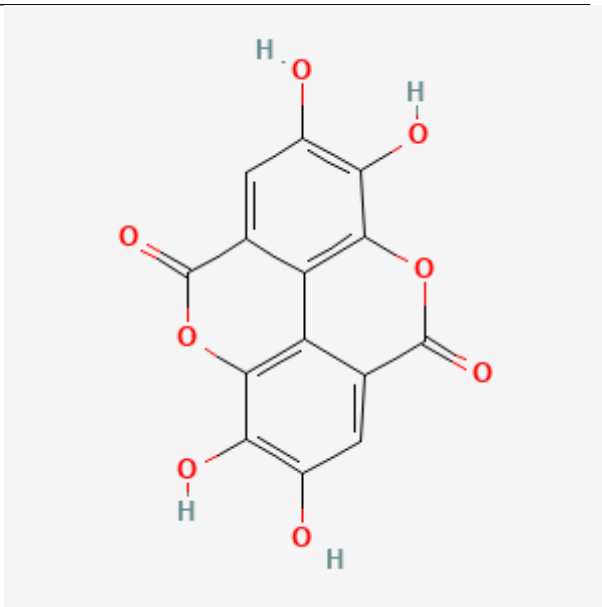
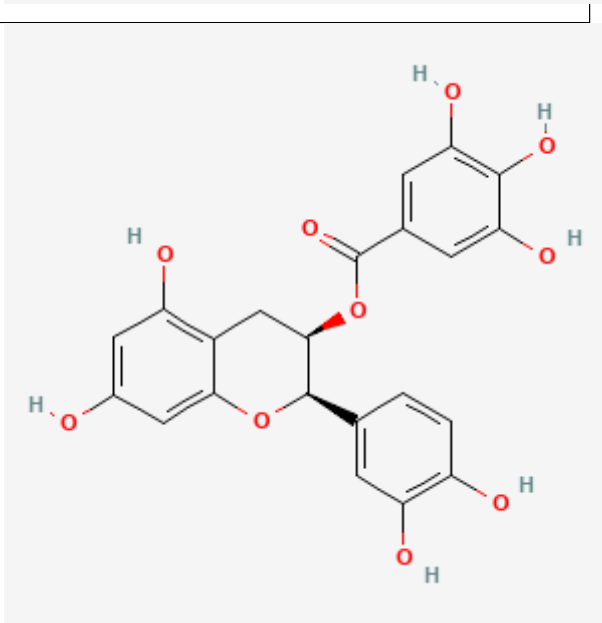
12. Carvacrol	
13. 2_hydroxy_chalcone	

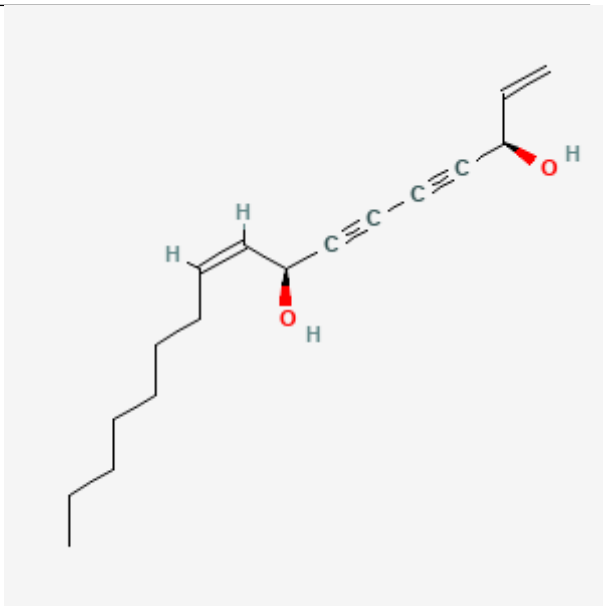
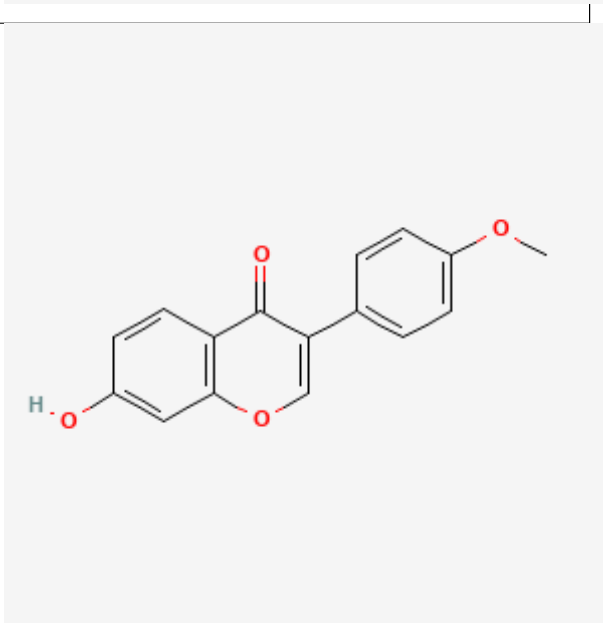
14. Catechin	
15. Citral	

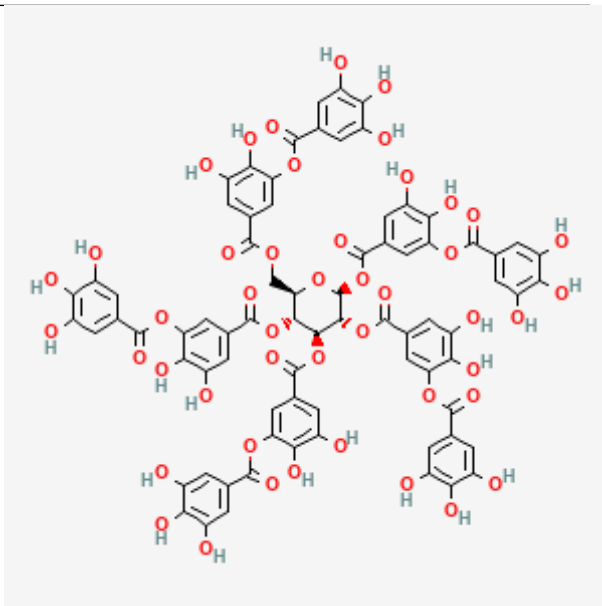
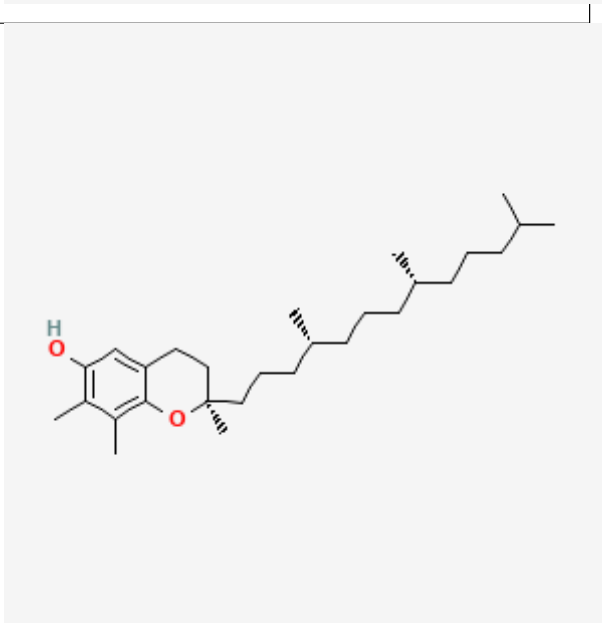
16. D9_Tetrahydrocannabinol	
17. Daidzein	

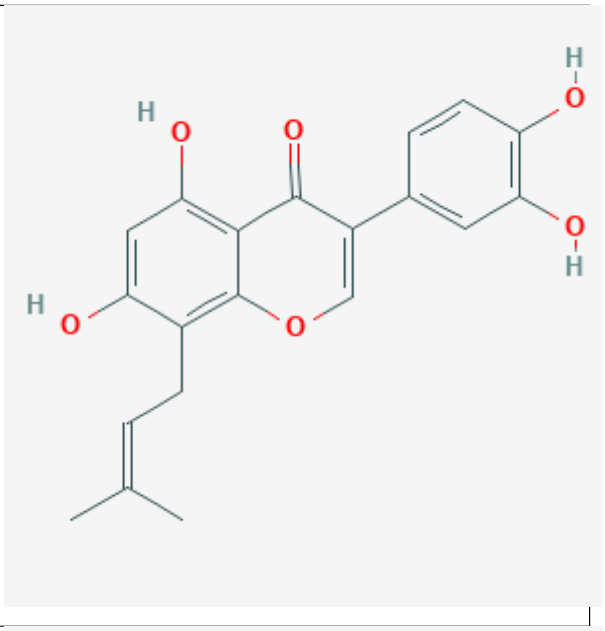
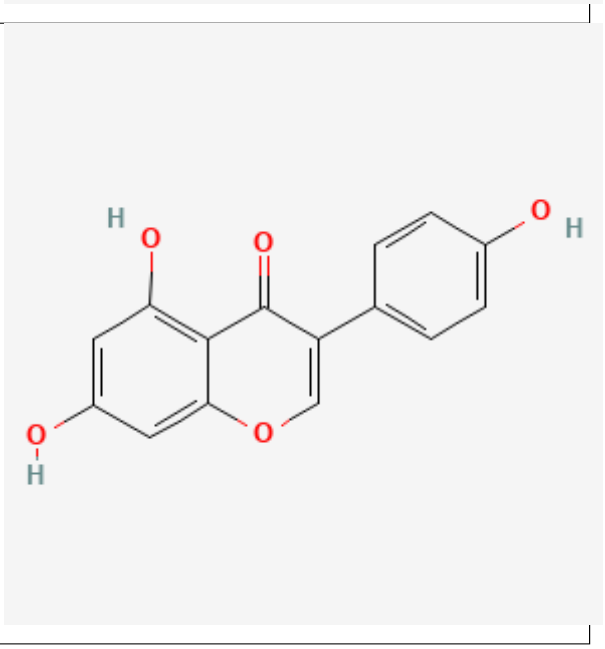
18. Dehydrotrametenolic_acid	
19. Delta_tocopherol	

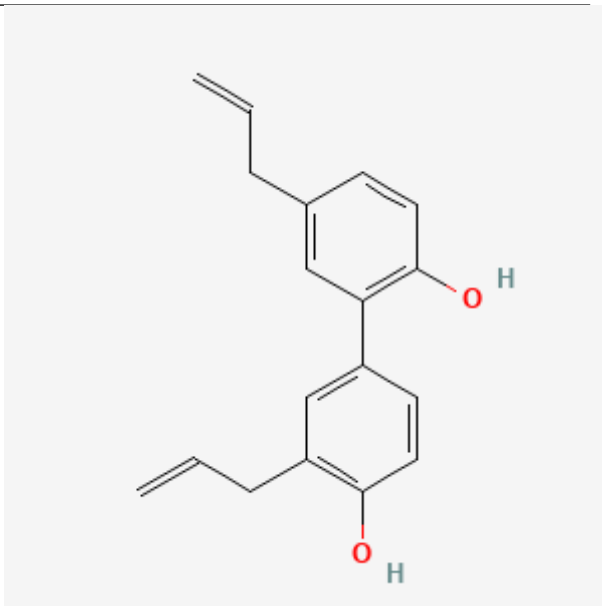
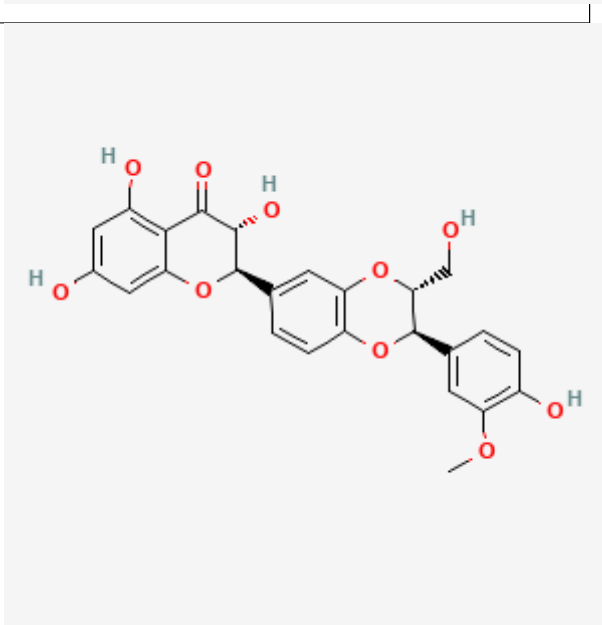
20. Deoxyelephantopin	
21. Echinatin	

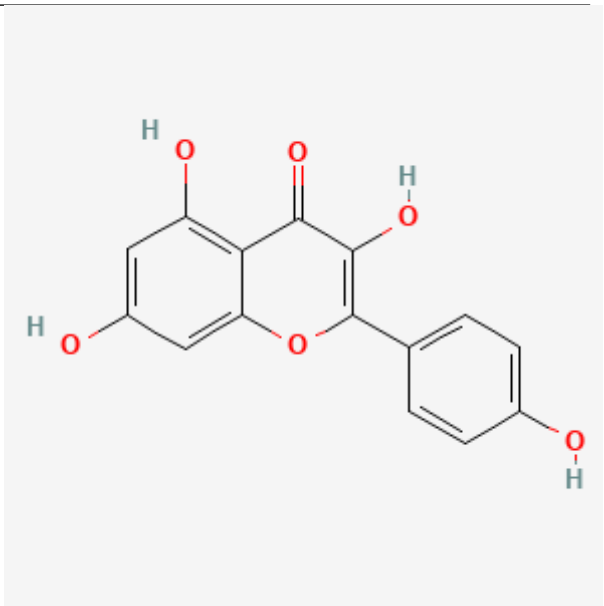
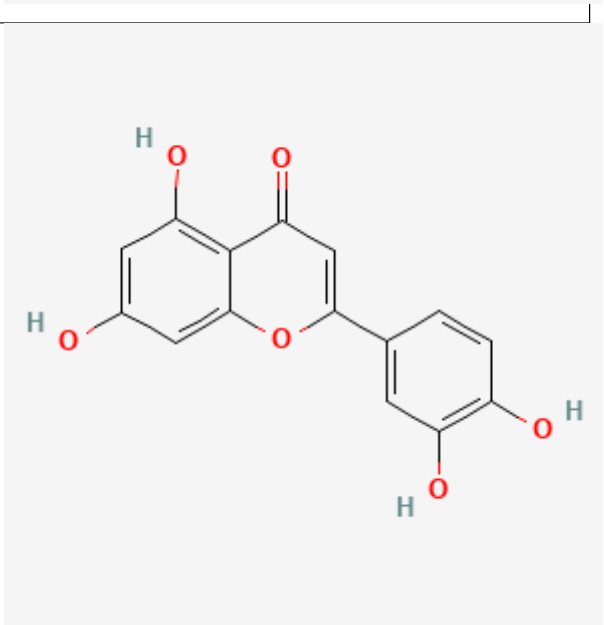
22. Ellagic_acid	
23. Epicatechin_gallate	

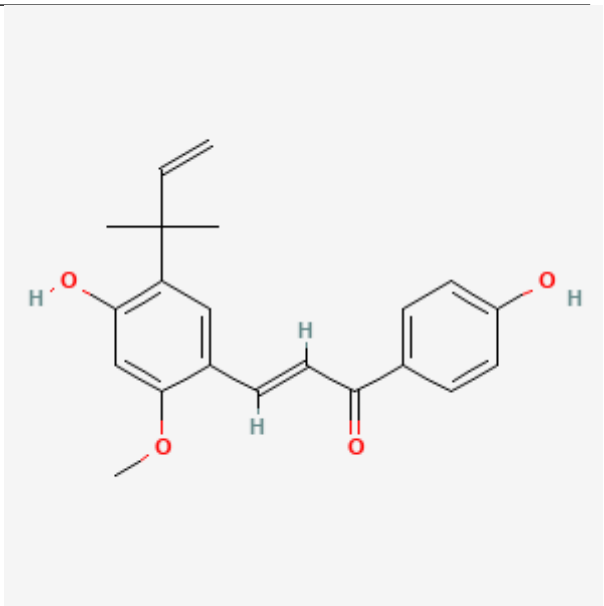
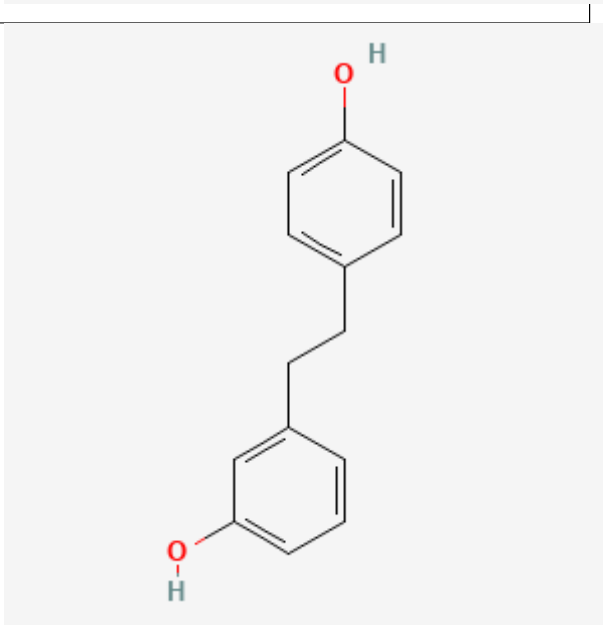
24. Falcarindiol	
25. Formononetin	

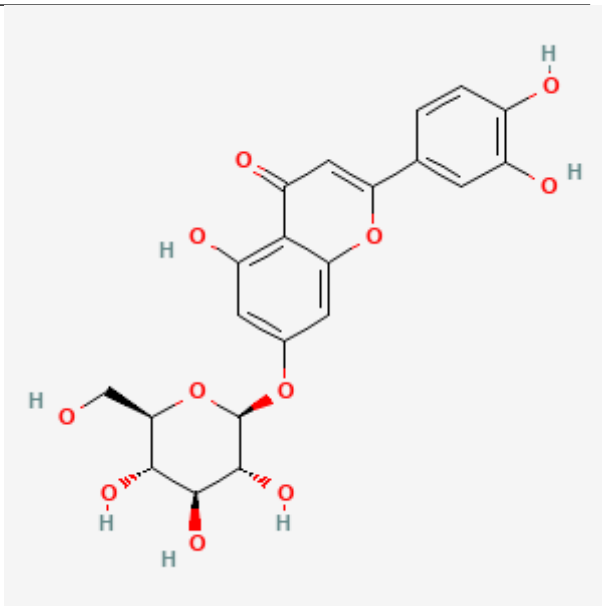
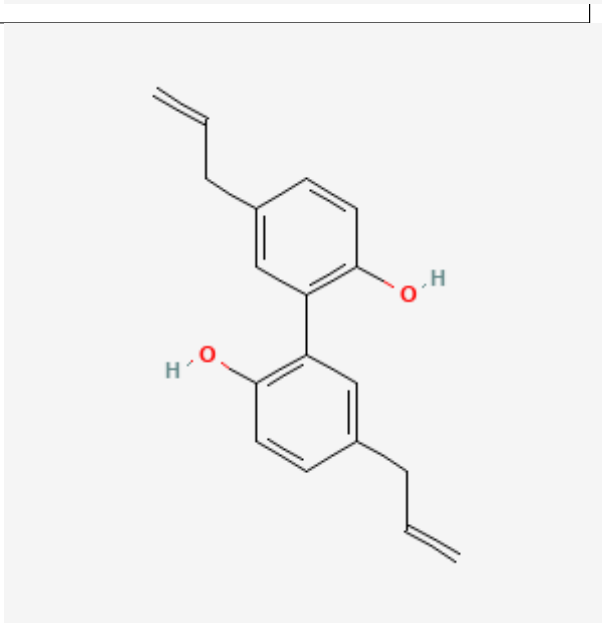
26. Gallotannins	
27. Gamma_tocopherol	

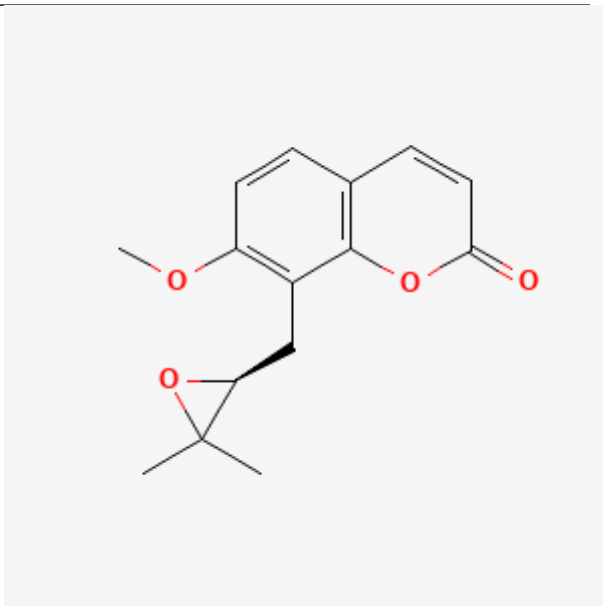
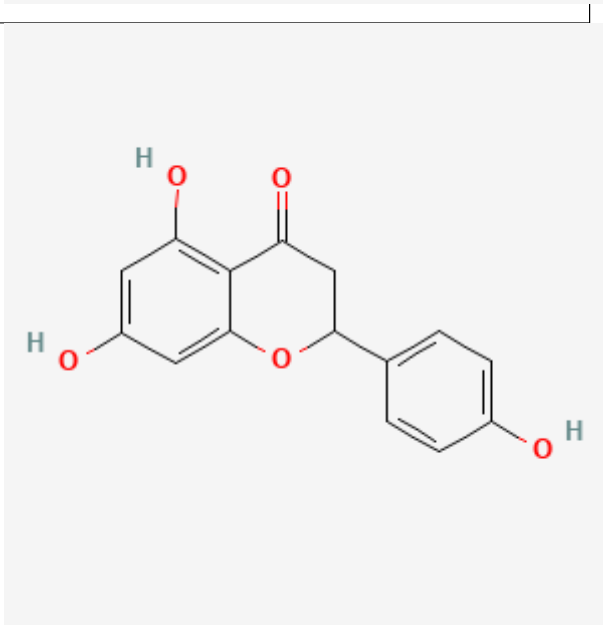
28. GancaoninL	
29. Genistein	

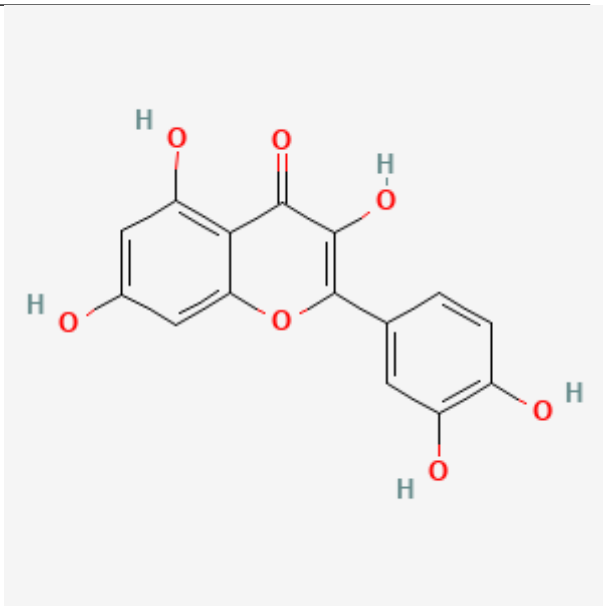
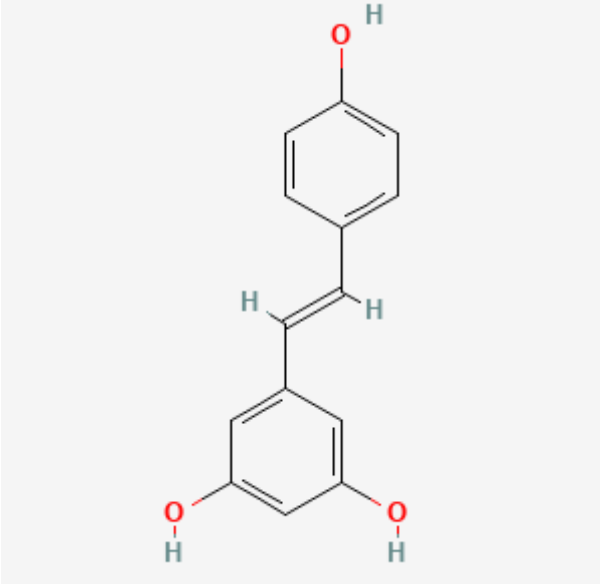
30. Honokiol	
31. Isosilybin_A	

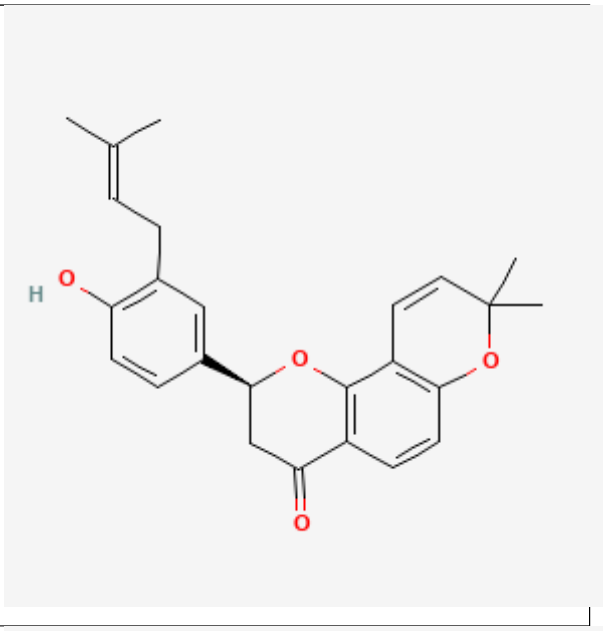
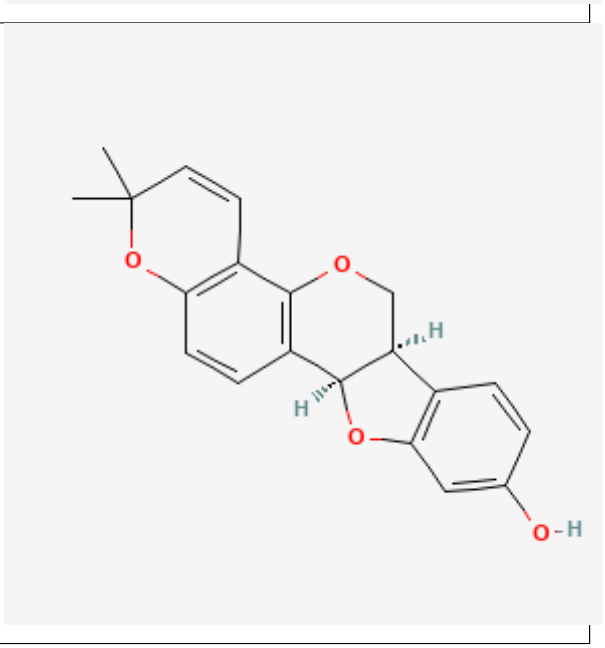
32. Kaempferol	
33. Luteolin	

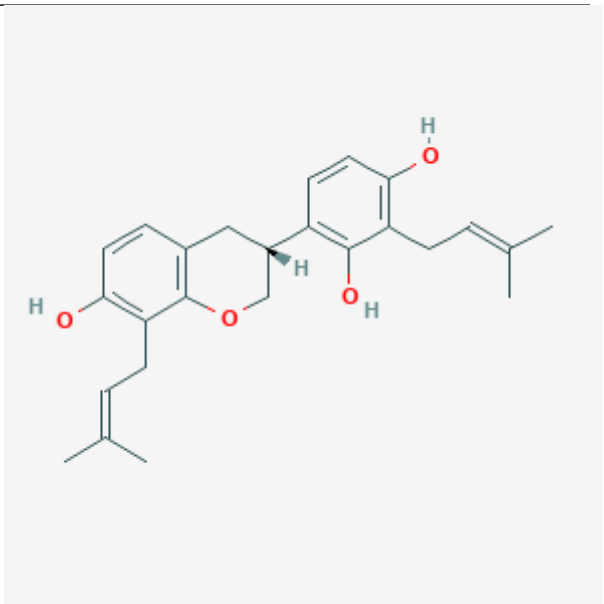
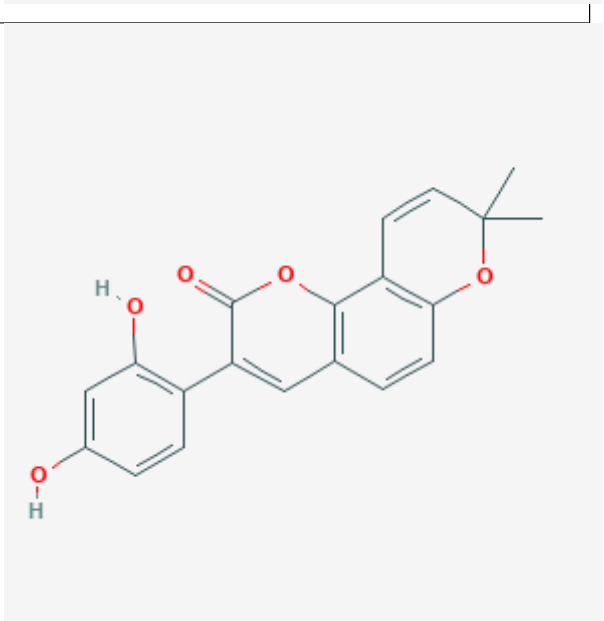
34. Licochalcone	
35. Lunularin	

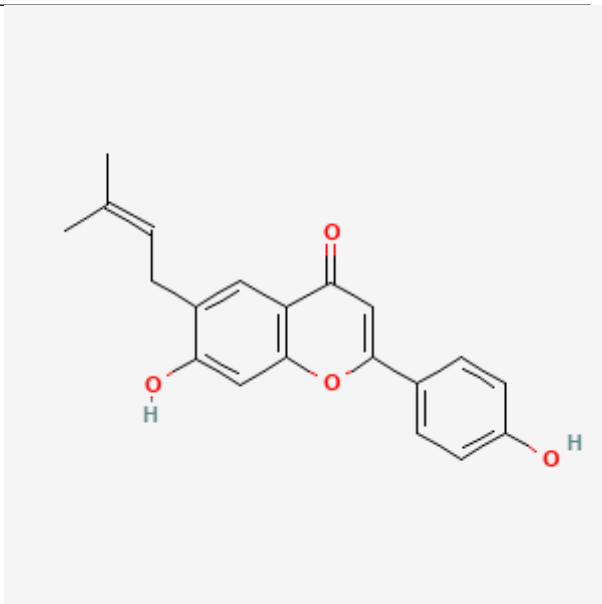
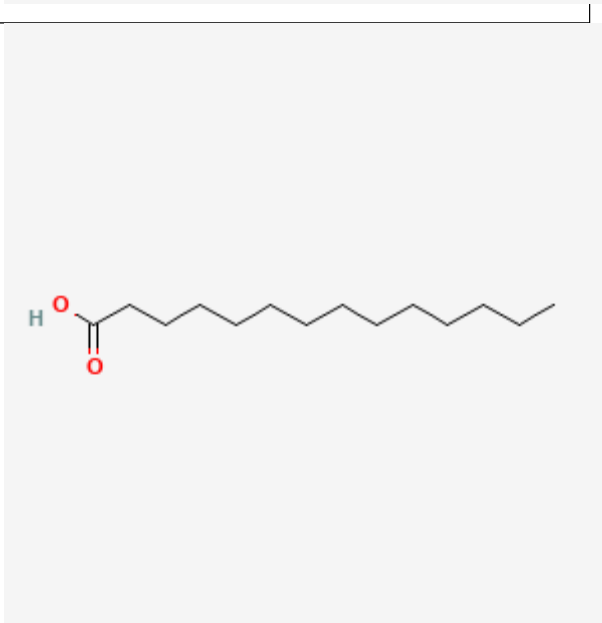
36. Luteoloside	
37. Magnolol	

38. Meranzin	
39. Naringenin	

40. Quercetin	
41. Resveratrol	

42. Shinflavanone	
43. Shinpterocarpin	

44. Kanzonol X	
45. Kanzonol W	

46. Licoflavanone A	
47. Myristic acid	

Then out of similarity search in PubChem database, 191 compounds were obtained which are given below:

Table 6.2: Showing obtained compounds after database similarity search.

Serial no.	Compound Name	Compound Id
1	Dihydrokaempferol	662
2	Heptadeca-1,9,16-trien-4,6-diyn-3-ol	1447
3	Orientanol B	1680
4	Acetovanillone	2214
5	Corylopsin	2356
6	Cannabinol	2543
7	Citropten	2775
8	Curcumin	2889
9	Dienestrol-[d4]	3049
10	Falcarinol	3322
11	(Rac)- Hesperitin	3593
12	Hexestrol	3606
13	Ipriflavone	3747
14	Isoformononetin	3764
15	Naringenin 7- Rhamnoglucoside	4441
16	Panaxydiol	4557
17	Oxybenzone	4632
18	Phlorizine	4789
19	Pomiferin	4871
20	Propofol	4943
21	Silymarin	5213
22	Vismiaphenone D	5675
23	BisphenolA	6623
24	Benzestrol	6827
25	Thymol	6989
26	Desaspidin	8238
27	Synhexyl	8334
28	Peucedanocoumarin I	10157
29	(+)-Samidin	10158
30	Cotoin	10175
31	Eugenin	10189
32	Osthole	10228
33	Carvacrol	10364
34	Olivetol	10377
35	Orcinol	10436
36	Hesperidin	10621
37	Durenol	10694
38	Primetin	11055
39	Paeonol	11092
40	4-Cumylphenol	11742
41	2-Octylphenol	13700
42	Phloraspidinol	15190
43	Phloraspine	15668
44	Ionol 6	15751
45	Dronabinol	16078

46	Isowogonin	20489
47	Cyasorb	20885
48	Naugawhite	24582
49	Pranchimgine	25785
50	Pedalitin	31161
51	Zingerone	31211
52	Silymarin	31553
53	Canbisol	41969
54	Lindleyin	42994
55	Cresol	50962
56	Ablukast	57109
57	Baicalin	64982
58	Salvin	65126
59	Xanthyletin	65188
60	Tinabinol	65443
61	Bergenin	66065
62	Xanthoxylin	66654
63	Pinocembrin	68071
64	Seselin	68229
65	Diresorcinol	68268
66	Peucenin	68477
67	Suberosin	68486
68	Methestrol	71620
69	Mexenone	71645
70	Bifluranol	71713
71	1-(2,4-dimethoxyphenyl)-3- (4-methoxyphenyl)prop-2- en-1 -one	71894
72	Hesperetin	72281
73	Magnolol	72300
74	Honokiol	72303
75	Anolignan B	72388
76	Suksdorfin	72414
77	GRb1	73148
78	Costunolide	73164
79	Pinostrobin	73201
80	Pinobanksin	73202
81	Xanthomicrol	73207
82	Alnetin	73210
83	Strictinin	73330
84	Corilagin	73568
85	Sakuranin	73607
86	Casuarictin	73644
87	Umuhengerin	73648
88	Eriocitrin	83489
89	Rosefuran	84825
90	Neopoucirin	85705
91	Thamnosmin	90218
92	Farrerol	91144
93	Vestitol	92503
94	Butin	92775

95	Prunin	92794
96	Quinidine gluconate	94328
97	Osajin	95168
98	Eupatorin	97214
99	Inulavosin	97820
100	Chamuvaritin	100418
101	Flavanomarein	101781
102	Procyanidin	107876
103	(S)-2,3-Dihydro-5,7- dihydroxy-6-methyl-2- phenyl-4-benzopyrone	114429
104	Liquiritigenin	114829
105	Tephrosin	114909
106	Astilbin	119258
107	Nepitrin	120742
108	Biphenyltriol	121794
109	Mallotojaponin	122659
110	Bavachinin A	122835
111	Lonchocarpol A	124035
112	Glabranin	124049
113	Glabridin	124052
114	Cyrtominetin	125309
115	Cristacarpin	126540
116	Sigmoidin D	129362
117	Angoletin	131162
118	Cornuside	131348
119	Selligueain A	132944
120	Alpinetin	154279
121	Kolaflavanone	155169
122	Spinosin	155692
123	Tephrinone	156589
124	Gambiriin C	156680
125	Isoferreirin	156743
126	Yuankanin	157000
127	Nupharin A	158198
128	Anguvetin	158308
129	Cirsimarin	159460
130	Pisiferic acid	162209
131	Glyceollin	162807
132	Irisolone	165103
133	Pectolinarin	168849
134	Irisflorentin	170569
135	Jujuboside A	171446
136	Roxbin B	176131
137	Coumarin	176970
138	Euphracal	182743
139	Crotmarine	185186
140	Gallin	191336
141	Ethanedione	226343
142	Phloroglucide	248349
143	Hamamelitannin	253775

144	Prodelphiniline	285704
145	Hippomanin A	323958
146	Ginsenoside rb1	432524
147	Betavulgarin	442668
148	Sayanedine	442820
149	Resveratrol	445154
150	1,3,6-tri-O-galloyl-beta-D- Glucose	452707
151	Spectrum4_001728	455260
152	2-methylhept-2-enal	534702
153	Kakuol	596894
154	Derrustone	629853
155	Citral	638011
156	Saurufuran A	643734
157	Neral	643779
158	Piceatannol	667639
159	Meranzin	1803558
160	Maackin	5279246
161	Quercetin	5280343
162	Formononetin	5280378
163	Pinosylvin	5280457
164	Farnesal	5280598
165	Kaempferol	5280863
166	Genistein	5280961
167	Falcarindiol	5281148
168	Bixin	5281226
169	Norbixin	5281249
170	Daidzein	5281708
171	Terminalin	5281711
172	6-Shogaol	5281794
173	Pseudobaptigenin	5281805
174	Ellagic acid	5281855
175	Licochalcone A	5318998
176	Hexenal	5352913
177	(3S,9Z)-heptadeca-1,9,16- trien-4,6-diyn-3-ol	5469785
178	Falcarinol	5469789
179	Deoxyelephantopin	6325056
180	Amorphastilbol	6440462
181	Echinatin	6442675
182	Sageone	6481824
183	Annatto	6537492
184	Epirosmanol	9884612
185	Ginsenoside Rb1	9898279
186	Shinpterocarpin	10336244
187	Isosilybin A	11059920
188	Glabrol	11596309
189	Furan	12224257
190	Rosmanol	13966122
191	Catechin-(4alpha->8)- gallocatechin-(4alpha->8)- catechin	131752348

6.2- Molecular docking study

Further after similarity search, all the 191 obtained compounds were docked against PPAR-γ receptor in Autodock vina. Docking results of all 191 compounds were obtained in Autodock Vina, dock scores are given in table 9. Protein molecule of PPAR-γ receptor was obtained from PDB (https://www.rcsb.org/) by ID 3V9T.Molecular docking is a method in drug designing to assess the binding confirmation of query compound in protein's active site (Grosdidier et al., 2011).High dockscore in molecular docking tells that compound binds very perfectly in active site of protein and thus it can activate the protein perfectly and can bring desired changes. Thus, compounds having highest dockscores in molecular docking were screened out and selected further for ADME studies in different softwares.

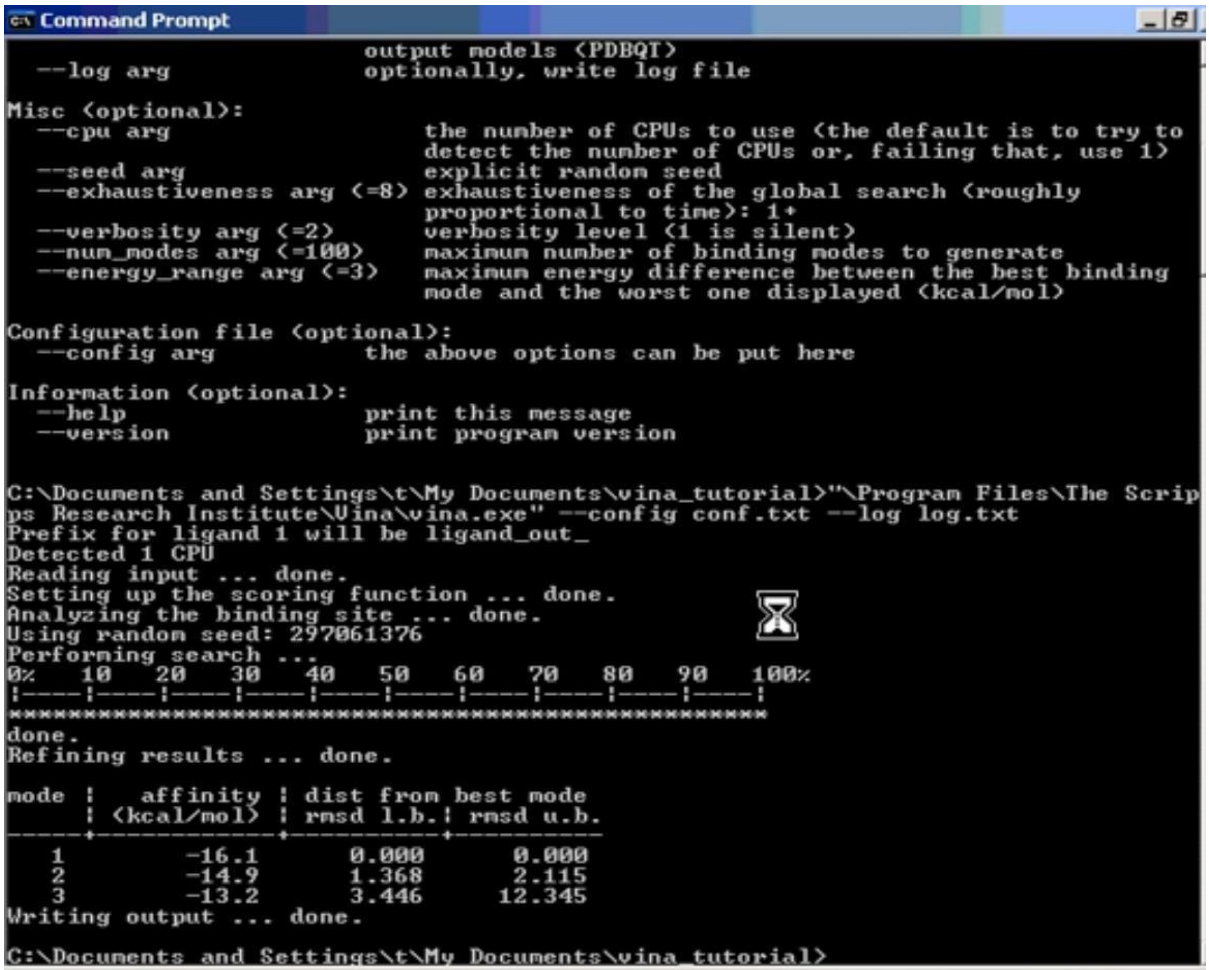


Figure 6.1: Showing docking result obtained after molecular docking in Autodock Vina

Table 6.3: Showing dock scores of all compounds after docking in Autodock vina.

Serial no.	Compound Name	Compound Id	Dockscore
1	Dihydrokaempferol	662	-8.1
2	Heptadeca-1,9,16-trien-4,6-diyn-3-ol	1447	-6.6
3	Orientanol B	1680	-9.1
4	Acetovanillone	2214	-5.9
5	Corylopsin	2356	-6.9
6	Cannabinol	2543	-7.9
7	Citropten	2775	-6.7
8	Curcumin	2889	-8.2
9	Dienestrol-[d4]	3049	-8.4
10	Falcarinol	3322	-5.9
11	(Rac)- Hesperitin	3593	-9.0
12	Hexestrol	3606	-8.2
13	Ipriflavone	3747	-8.2
14	Isoformononetin	3764	-8.0
15	Naringenin 7-Rhamnoglucoside	4441	-9.9
16	Panaxydiol	4557	-6.6
17	Oxybenzone	4632	-7.2
18	Phlorizine	4789	-8.3
19	Pomiferin	4871	-9.3
20	Propofol	4943	-6.9
21	Silymarin	5213	-10.2
22	Vismiaphenone D	5675	-8.4
23	BisphenolA	6623	-7.3
24	Benzestrol	6827	-7.3
25	Thymol	6989	-5.9
26	Desaspidin	8238	-7.0
27	Synhexyl	8334	-8.0
28	Peucedanocoumarin I	10157	-6.7
29	(+)-Samidin	10158	-9.3

30	Cotoin	10175	-6.9
31	Eugenin	10189	-6.7
32	Osthole	10228	-7.7
33	Carvacrol	10364	-5.8
34	Olivetol	10377	-5.9
35	Orcinol	10436	-4.9
36	Hesperidin	10621	-10.6
37	Durenol	10694	-6.1
38	Primetin	11055	-7.6
39	Paeonol	11092	-5.8
40	4-Cumylphenol	11742	-6.0
41	2-Octylphenol	13700	-5.8
42	Phloraspidinol	15190	-6.8
43	Phloraspine	15668	-6.8
44	Ionol 6	15751	-8.3
45	Dronabinol	16078	-7.9
46	Isowogonin	20489	-9.0
47	Cyasorb	20885	-7.7
48	Naugawhite	24582	-7.1
49	Pranchimgine	25785	-9.0
50	Pedalitin	31161	-8.8
51	Zingerone	31211	-6.6
52	Silymarin	31553	-9.7
53	Canbisol	41969	-7.9
54	Lindleyin	42994	-8.5
55	Cresol	50962	-8.8
56	Ablukast	57109	-9.1
57	Baicalin	64982	-9.4
58	Salvin	65126	-7.5
59	Xanthyletin	65188	-7.5
60	Tinabinol	65443	-8.5
61	Bergenin	66065	-7.2

62	Xanthoxylin	66654	-5.9
63	Pinocembrin	68071	-8.6
64	Seselin	68229	-7.5
65	Diresorcinol	68268	-7.0
66	Peucenin	68477	-7.3
67	Suberosin	68486	-7.7
68	Methestrol	71620	-9.0
69	Mexenone	71645	-7.3
70	Bifluranol	71713	-8.1
71	1-(2,4-dimethoxyphenyl)-3- (4-methoxyphenyl)prop-2-en- 1-one	71894	-7.9
72	Hesperetin	72281	-7.9
73	Magnolol	72300	-7.6
74	Honokiol	72303	-8.0
75	Anolignan B	72388	-7.9
76	Suksdorfin	72414	-7.0
77	GRb1	73148	-8.5
78	Costunolide	73164	-6.7
79	Pinostrobin	73201	-8.7
80	Pinobanksin	73202	-8.9
81	Xanthomicrol	73207	-7.9
82	Alnetin	73210	-9.1
83	Strictinin	73330	-8.4
84	Corilagin	73568	-9.5
85	Sakuranin	73607	-7.4
86	Casuarictin	73644	-8.8
87	Umuhengerin	73648	-8.2
88	Eriocitrin	83489	-9.2
89	Rosefuran	84825	-5.7
90	Neopoucirin	85705	-10.6
91	Thamnosmin	90218	-8.1

92	Farrerol	91144	-8.4
93	Vestitol	92503	-8.2
94	Butin	92775	-8.6
95	Prunin	92794	-8.5
96	Quinidine gluconate	94328	-4.3
97	Osajin	95168	-9.4
98	Eupatorin	97214	-7.7
99	Inulavosin	97820	-8.0
100	Chamuvaritin	100418	-10.4
101	Flavanomarein	101781	-9.1
102	Procyanidin	107876	-9.5
103	(S)-2,3-Dihydro-5,7- dihydroxy-6-methyl-2- phenyl-4-benzopyrone	114429	-8.2
104	Liquiritigenin	114829	-6.6
105	Tephrosin	114909	-8.5
106	Astilbin	119258	-9.5
107	Nepitrin	120742	-9.9
108	Biphenyltriol	121794	-7.1
109	Mallotojaponin	122659	-7.7
110	Bavachinin A	122835	-9.6
111	Lonchocarpol A	124035	-9.6
112	Glabranin	124049	-9.4
113	Glabridin	124052	-9.6
114	Cyrtominetin	125309	-8.4
115	Cristacarpin	126540	-9.0
116	Sigmoidin D	129362	-8.8
117	Angoletin	131162	-8.6
118	Cornuside	131348	-8.5
119	Selligueain A	132944	-10.2
120	Alpinetin	154279	-7.4
121	Kolaflavanone	155169	-8.5

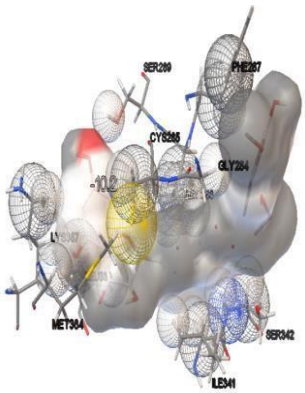
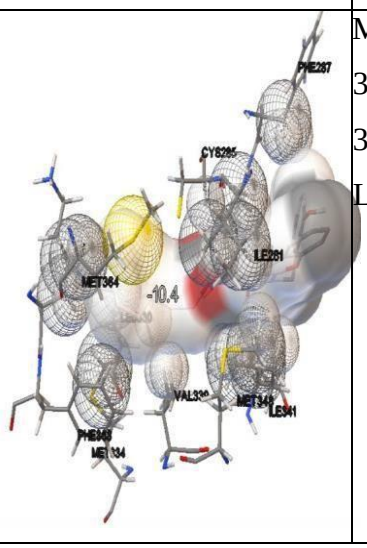
122	Spinosin	155692	-8.0
123	Tephrinone	156589	-9.5
124	Gambiriin C	156680	-9.2
125	Isoferreirin	156743	-7.3
126	Yuankanin	157000	-9.2
127	Nupharin A	158198	-9.8
128	Anguvetin	158308	-8.3
129	Cirsimarin	159460	-8.3
130	Pisiferic acid	162209	-6.6
131	Glyceollin	162807	-8.1
132	Irisolone	165103	-8.6
133	Pectolinarin	168849	-10.1
134	Irisflorentin	170569	-7.7
135	Jujuboside A	171446	-10.1
136	Roxbin B	176131	-9.2
137	Coumarin	176970	-8.9
138	Euphracal	182743	-7.8
139	Crotmarine	185186	-9.4
140	Gallin	191336	-8.6
141	Ethanedione	226343	-7.4
142	Phloroglucide	248349	-6.7
143	Hamamelitannin	253775	-7.4
144	Prodelphiniline	285704	-9.2
145	Hippomanin A	323958	-9.0
146	Ginsenoside rb1	432524	-9.0
147	Betavulgarin	442668	-7.9
148	Sayanedine	442820	-7.9
149	Resveratrol	445154	-7.3
150	1,3,6-tri-O-galloyl-beta-D-glucose	452707	-9.4
151	Spectrum4_001728	455260	-7.3
152	2-methylhept-2-enal	534702	-4.7

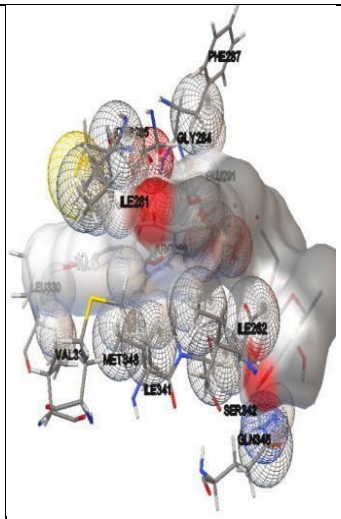
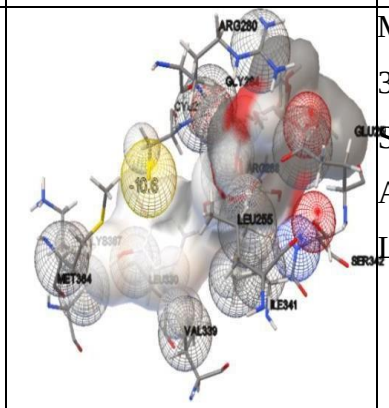
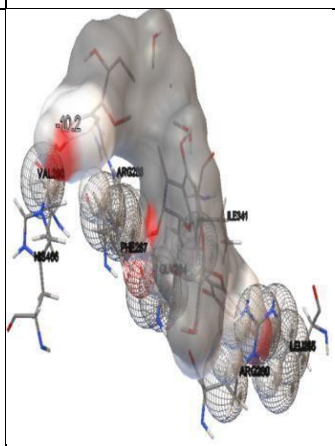
153	Kakuol	596894	-7.0
154	Derrustone	629853	-8.6
155	Citral	638011	-5.4
156	Saurufuran A	643734	-8.1
157	Neral	643779	-5.6
158	Piceatannol	667639	-7.4
159	Meranzin	1803558	-7.5
160	Maackin	5279246	-8.5
161	Quercetin	5280343	-8.1
162	Formononetin	5280378	-8.3
163	Pinosylvin	5280457	-7.6
164	Farnesal	5280598	-6.7
165	Kaempferol	5280863	-7.7
166	Genistein	5280961	-7.9
167	Falcarindiol	5281148	-6.2
168	Bixin	5281226	-9.0
169	Norbixin	5281249	-8.1
170	Daidzein	5281708	-8.2
171	Terminalin	5281711	-10.2
172	6-Shogaol	5281794	-6.6
173	Pseudobaptigenin	5281805	-8.4
174	Ellagic acid	5281855	-8.0
175	Licochalcone A	5318998	-8.1
176	Hexenal	5352913	-5.1
177	(3S,9Z)-heptadeca-1,9,16-trien-4,6-diyn-3-ol	5469785	-6.3
178	Falcarinol	5469789	-6.4
179	Deoxyelephantopin	6325056	-8.3
180	Amorphastilbol	6440462	-9.7
181	Echinatin	6442675	-8.9
182	Sageone	6481824	-8.0
183	Annatto	6537492	-8.3
184	Epirosmanol	9884612	-8.1

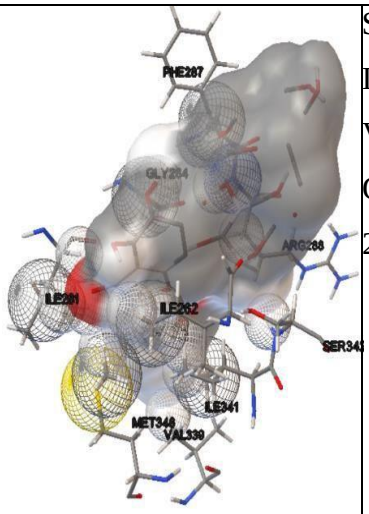
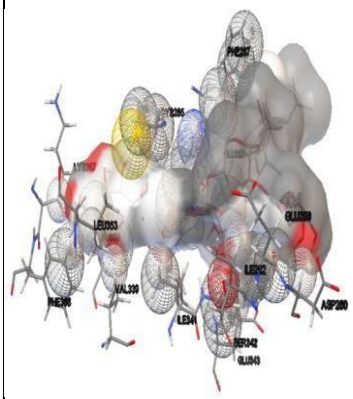
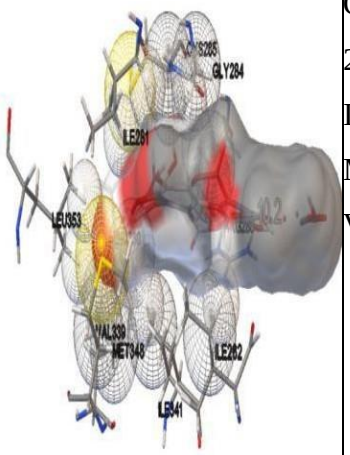
185	Ginsenoside Rb1	9898279	-7.9
186	Shinpterocarpin	10336244	-8.2
187	Isosilybin A	11059920	-9.0
188	Glabrol	11596309	-10.2
189	Furan	12224257	-4.9
190	Rosmanol	13966122	-7.3
191	Catechin-(4alpha->8)-gallocatechin-(4alpha->8)-Catechin	131752348	-9.8

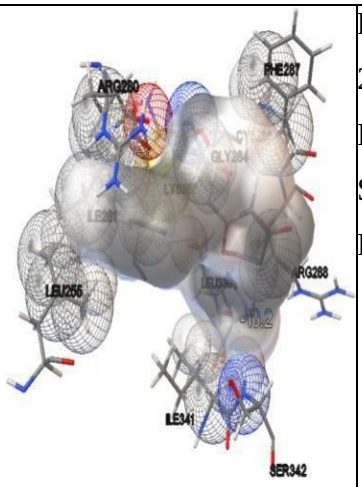
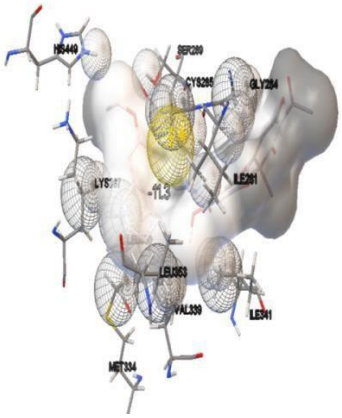
After docking results analysis, only 9 compounds which had highest (most negative) dock score (more than 10) were selected for further ADMET study, whereas those molecules having less than 10 dock score were rejected. The standard compound (x-ray crystallized ligand) was having dock score -11.3. The dock scores, docking pictures and docking interactions of selected 9 compounds areenlisted in the table 10.

Table 6.4: Showing dock score and docking interactions of compounds having highest dock score.

Names Of searched compounds having highest dock score	Pubchem Id	Dock score (kcal/mol)	Docking picture	Docking interactions
Silymarin	5213	-10.2		MET364, ILE341, SER342, CYS285, SER289, PHE287, GLY284, ARG288, LEU 330, LYS367
Chamuvariti n	100418	-10.4		MET364,PHE368,MET334,LYS367,MET348,VAL339, CYS285,PHE287, LEU330, ILE341

Neopoucirin	85705	-10.6		MET364, ILE341, SER342, LYS367, LEU330, ARG 288, CYS285, SER289, GLY284, PHE287,
Hesperidin	10621	-10.6		MET364,LYS367,VAL 339, LEU255,GLU259, SER342, CYS285, ARG280,ILE341, ARG288, LEU330, GLY284
Selligueain A	132944	-10.2		ILE341, LEU255, ARG288, GLY284, ARG280, PHE 287, VAL290, HIS466,

Pectolinarin	168849	-10.1		SER342, ILE262, ILE341, MET348, VAL339, ILE281, GLY284, ARG288, PHE 287
Jujuboside A	171446	-10.1		LYS367,LEU330, PHE368, VAL339,ARG288,LEU 353, CYS285, ILE341, SER342, GLU343,ILE262, GLU259, ASP260, GLU291, PHE287
Terminalin	528171 1	-10.2		CYS285, GLY284, ARG 288, ILE281, LEU330, ILE262, LEU353, MET348, ILE3 41, VAL339

Glabrol	11596309	-10.2		PHE287, CYS285,ARG 288, GLY284, ARG280, LYS 367, ILE281, SER342, ILE341, LEU255, LEU330
X-ray ligand docked with PPAR-γ receptor		-11.3		SER289, HIS449, CYS285,GLY284, LYS364,LEU 330, ILE281, ILE341, VAL339, LEU353, MET334

Docking of PPAR-γ receptor with x-ray crystallized ligand i.e. cercosporamide derivative with dock score 11.3 and Hesperidin with dock score 10.6 are shown below as this was found most specific among all others:

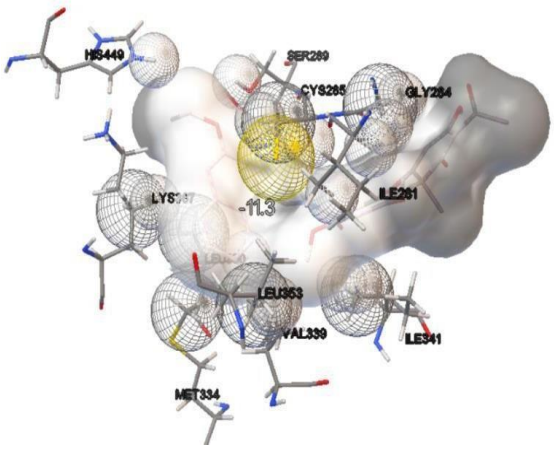


Figure 6.2: Showing docking ofX-ray receptor ligand docked with PPAR-γ receptor

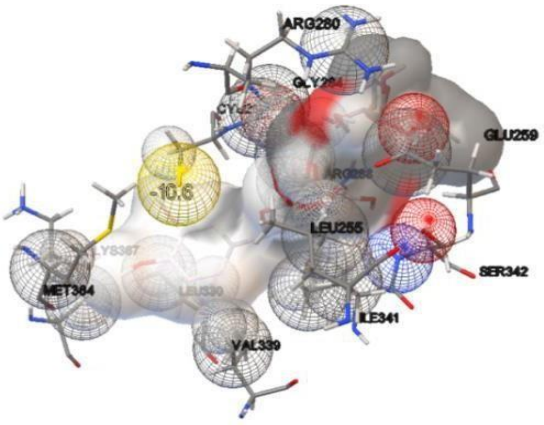


Figure 6.3: Showing docking of Hesperidin with PPAR-γ

It was observed from docking studies against PPAR-γ receptor, that as all 9 docked compounds have highest (most negative) dock Score, close to dock score of cercosporamide (-11.3), the x-ray crystallized ligand of PPAR-γ receptor, thus it can be said that all 9 docked compounds act as agonist of PPAR-γ receptor. However, antagonists are supposed to have lowest dock score (least negative) value in comparison with standard. Moreover, it was also observed that cercosporamide derivative formed hydrogen bond interactions with Ser 289, which is found to be important for binding with PPAR-γ agonists also. The best 9 docked compounds formed H-bonding interactions with Ser342 and Ser289 of PPAR-γ receptor via hydrogen bonding. In addition, it was also reported that an H-bonding interaction with Ser342 in PPAR-gamma is important for partial agonism. Our compounds were also found to have hydrogen bonding interactions with Ser289 and Ser342. Thus, it can be said that all 9 screened compounds can act as partial agonists against PPAR-gamma receptor.

6.3- ADME study:

The 9 best docking compounds were subjected for ADME studies in different tools (swiss ADME, vNN ADMET, admetSAR, DRULITO and ADMETlab). All the ADME studies results are given in Table 11, 12, 13, 14 and 15. Only 17 common parameters were used for analysis of result obtained from all 5 different softwares to compare the results of these softwares collectively, which is given in table 16, whereas table 17 shows normalrange of values of different parameters used in ADME analysis. On the basis of table 17, table 16 was obtained.

After process of molecular docking, selected 9 compounds of highest dock score underwent ADME analysis using 5 different softwares namely SwissADME, vNN ADMET, admetSAR, DRULITO and ADMETlab.The result of all these softwares is given below:

6.3.1- SwissADME:

It is a free web tool for physiochemical properties and pharmacokinetic evaluation of a drug to predict its druglikeness and to assess its medicinal chemistry friendliness. A potent drug reaches to the body tissues in sufficient concentration and should stay in body till expected biological event occurs. Thus, ADME (absorption, distribution, metabolism and excretion) studies become essentially required to limit down lead compound when there are numerous compounds in consideration. The methods used by Swiss ADME are iLogP, Boiled Egg and bioavailability radar.

Table-6.5: Showing result of SwissADME software

Properties	**Silymarin**	**Chamuvaritin**	**Neopoucirin**	**Hesperidin**	**Selligueain A**	**Pectolinarin**	**Jujuboside A**	**Terminalin**	**Glabrol**
Canonical SMILES	OCC1Oc2ccc(cc2OC1c1ccc(c(c1)OC)O)C1Oc2cc(O)cc(c2C(=O)C1O)O	COc1ccc(cc1O)C1CC(=O)c2c(O1)cc(cc2O)OC1OC(COC2OC(C)C(C(C2O)O)O)C(C(C1O)O)O	COc1ccc(cc1)C1CC(=O)c2c(O1)cc(cc2O)OC1OC(COC2OC(C)C(C(C2O)O)O)C(C(C1O)O)O	O=C(c1c(O)c(Cc2ccccc2O)c2c(c1O)Cc1c(O2)cccc1)CCc1ccccc1	Oc1ccc(cc1)C1Oc2c(C(C1O)c1c(O)cc(c3c1OC(C(C3)O)c1ccc(cc1)O)O)c(O)cc1c2C2c3c(O)cc(cc3OC(O1)(C2O)c1ccc(cc1)O)O	COc1c(OC2OC(COC3OC(C)C(C(C3O)O)O)C(C(C2O)O)O)cc2c(c1O)c(=O)cc(o2)c1ccc(cc1)OC	OCC1OC(OCC2OC(OC3C(O)COC(C3OC3OC(C)C(C(C3O)O)O)OC3CCC4(C(C3(C)C)CCC3(C4CCC4C53COC3(C5)C4C(C)(O)CC(O3)C=C(C)C)C)C(C(C2O)O)OC2OCC(C(C2O)O)O)C(C(C1O)O)O	Oc1cc2c(=O)oc3c(c2c(c1O)O)c1c(=O)oc2c4c1c(c3O)oc(=O)c4c1c(c2O)oc(=O)c2c1c(O)c(O)c(c2)O	CC(=CCc1cc(ccc1O)C1CC(=O)c2c(O1)c(CC=C(C)C)c(cc2)O)C
Formula	C25H22O10	C28H34O15	C28H34O14	C29H24O5	C45H36O15	C29H34O15	C58H94O26	C28H10O16	C25H28O4
MW	482.44	610.56	594.56	452.5	816.76	622.57	1207.35	602.37	392.49
#Heavy atoms	35	43	42	34	60	44	84	44	29
#Aromatic heavy atoms	18	12	12	24	36	16	0	32	12
Fraction Csp3	0.24	0.54	0.54	0.14	0.2	0.48	0.97	0	0.32
Rotatable bonds	4	7	7	6	4	8	13	0	5

#H-bond acceptors	10	15	14	5	15	15	26	16	4
#H-bond donors	5	8	7	3	11	7	14	8	2
MR	120.55	141.41	139.38	130.98	210.45	148.29	285.51	150.5	116.99
TPSA	155.14	234.29	214.06	86.99	259.45	227.2	393.98	282.68	66.76
iLOGP	2.79	2.6	3.1	3	2.74	1.73	2.97	0.75	3.87
XLOGP3	1.9	-0.14	-0.66	6.43	4.4	0.47	-1.62	1.91	6.05
WLOGP	1.71	-1.48	-1.19	5.91	4.37	-0.79	-2.72	2.65	5.5
MLOGP	-0.4	-3.04	-2.57	3.6	0.68	-3.03	-5.03	-0.31	3.38
Silico s-IT Log P	1.92	-1.55	-1.08	6.29	2.92	-0.53	-3.69	2.67	6.02
Consensus Log P	1.59	-0.72	-0.48	5.04	3.02	-0.43	-2.02	1.53	4.96
ESOL Log S	-4.14	-3.28	-2.86	-6.82	-7.86	-3.74	-5.45	-5.32	-6.06
ESOL Solubility (mg/ml)	3.46E-02	3.19E-01	8.21E-01	6.81E-05	1.14E-05	1.14E-01	4.31E-03	2.91E-03	3.41E-04
ESOL Solubility (mol/l)	7.17E-05	5.23E-04	1.38E-03	1.50E-07	1.39E-08	1.83E-04	3.57E-06	4.83E-06	8.69E-07
ESOL Class	Moderately soluble	Soluble	Soluble	Poorly soluble	Poorly soluble	Soluble	Moderately soluble	Moderately solubl e	Poorly solubl e
Ali Log S	-4.78	-4.33	-3.36	-8.05	-9.57	-4.81	-6.14	-7.47	-7.23
Ali Solubility (mg/ml)	7.99E-03	2.88E-02	2.59E-01	4.03E-06	2.22E-07	9.64E-03	8.67E-04	2.04E-05	2.30E-05
Ali Solubility (mol/l	1.66E-05	4.72E-05	4.35E-04	8.90E-09	2.72E-10	1.55E-05	7.18E-07	3.39E-08	5.87E-08

)									
Ali Class	Moderately soluble	Moderately soluble	Soluble	Poorly soluble	Poorly soluble	Moderately solubl e	Poorly soluble	Poorly solubl e	Poorl y solubl e
Silico s-IT LogS w	-4.5	-0.58	-1.18	-9.27	-7.85	-2.25	2.62	-6.54	-6.46
Silico s-IT Solubility (mg/ml)	1.53E-02	1.60E+02	3.96E+01	2.42E-07	1.15E-05	3.48E+00	4.99E+05	1.76E-04	1.36E-04
Silico s-IT Solubility (mol/l)	3.16E-05	2.62E-01	6.65E-02	5.36E-10	1.41E-08	5.60E-03	4.13E+02	2.92E-07	3.47E-07
Silico s-IT class	Moderately soluble	Soluble	Soluble	Poorly soluble	Poorly soluble	Soluble	Soluble	Poorly solubl e	Poorl y solubl e
GI absorption	Low	Low	Low	High	Low	Low	Low	Low	High
BBB permeant	No	No	No	No	No	No	No	No	No
Pgp substrate	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No
CYP1A2 inhibitor	No	No	No	No	No	No	No	No	No
CYP2C19 inhibitor	No	No	No	Yes	No	No	No	No	Yes
CYP2C9 inhibitor	No	No	No	No	Yes	No	No	No	Yes
CYP2D6 inhibitor	No	No	No	Yes	No	No	No	No	No
CYP3A4	Yes	No	No	No	No	No	No	No	Yes

inhibitor									
log Kp (cm/s)	-7.89	-10.12	-10.4	-4.49	-8.16	-9.76	-14.82	-8.62	-4.4
Lipinski #violations	0	3	3	0	3	3	3	3	0
Ghose #violations	1	4	4	2	3	4	4	2	0
Veber #violations	1	1	1	0	1	1	2	1	0
Egan #violations	1	1	1	1	1	1	1	1	0
Muegge #violations	1	4	3	1	5	4	5	5	1
Bioavailability Score	0.55	0.17	0.17	0.55	0.17	0.17	0.17	0.17	0.55
PAINS #alerts	0	0	0	0	0	0	0	1	0
Brenk #alerts	0	0	0	0	0	0	2	4	1
Leadlikeness #violations	1	1	1	2	2	2	2	1	2
Synthetic Accessibility	4.92	6.34	6.27	3.62	7.3	6.63	10	4.19	4.15

6.3.2- admetSAR:

It is an open source, comprehensive database for prediction of ADME properties of compounds. It uses QSAR (Quatitative structure activity relatioship), qualitative classification and quantitative regression model for prediction of ADME properties. These models include blood-brain barrier penetration, human intestinal absorption, P-glycoprotein substrate and inhibitor, Caco-2 permeability, CYP450 substrate and inhibitor (CYP1A2, 2C9, 2D6, 2C19, and 3A4), AMES mutagenicity, hERG inhibitors, carcinogens, honey bee toxicity, fathead minnow toxicity and Tetrahymena Pyriformis toxicity. Smile sequence of compounds was used as a query in this database to obtain following result:

Table 6.6: Showing result of admetSAR software:

Properties	Silymarin	Chamuvaritin	Neopoucirin	Hesperidin	Selligueain A	Pectolinarin	Jujuboside A	Terminalin	Glabrol
Molecular Weight	482.44	610.57	594.57	452.51	816.77	622.58	1207.36	602.37	392.50
AlogP	2.36	-1.16	-0.86	5.91	5.13	-0.79	-2.72	2.65	5.82
H-Bond Acceptor	10	15	14	5	15	15	26	16	4
H-Bond Donor	5	8	7	3	11	7	14	8	2
Rotatable Bonds	4	7	7	6	4	8	13	0	5
Human Intestinal Absorption	+0.9884	+0.8161	+0.8106.81	6+10.9881	+0.9570	+0.7710	- 0.5716	+0.9815	+0.9954
Caco-2	- 0.8574	- 0.8816	-0.8707	-0.8165	- 0.8911	- 0.8758	- 0.8796	-0.8846	+0.6179
Blood Brain Barrier	- 0.6573	- 0.9570	-0.9570	-0.3523	- 0.2652	- 0.9570	- 0.3269	-0.6027	-0.5358
Human oral bioavailability	- 0.7571	- 0.8286	-0.8571	-0.6714	- 0.7714	- 0.7143	- 0.7571	+0.7286	-0.6143
Subcellular	Mitochondria	Mitochondria	Mitochondria	Mitochondria	Mitochondria	Mitochondr	Mitochondria	Mitochondria	Mitochondria

localzatio n	0.6746	0.6902	0.6902	0.7971	0.5335	ia 0.6680	0.7487	0.5553	0.8330
OATP2B 1 inhibitior	- 0.5684	- 0.8556	-0.8560	-0.5689	- 0.7112	- 0.5742	- 1.0000	-0.5542	-0.5835
OATP1B 1 inhibitior	+0.9413	+0.9414	+0.9284	+0.8551	+0.7533	- 0.7738	+0.8251	+0.9084	+0.8986
OATP1B 3 inhibitior	+0.8605	+0.9479	+0.9479	+0.8945	- 0.6091	+0.9137	+0.8885	+0.9673	+0.9277
MATE1 inhibitior	- 0.8600	- 0.9600	-0.9600	-0.8600	- 0.8800	- 0.8600	- 0.9800	-0.8400	-0.9800
OCT2 inhibitior	- 0.8750	- 0.8250	-0.8250	-0.8000	- 0.9250	- 0.8000	- 0.5500	-0.9000	-0.9000
BSEP inhibitior	+0.9071	+0.7312	+0.7749	+0.9402	+0.9391	+0.8031	+0.8449	-0.6580	+0.9239
P-glycoprotein inhibitior	+0.9511	- 0.9166	-0.8489	+0.7498	+0.7279	- 0.6881	+0.7461	-0.6070	+0.7841
P-glycoprotein substrate	- 0.8633	- 0.5059	-0.6365	-0.6837	- 0.5492	+0.6242	+0.6409	-0.9531	-0.7519
CYP3A4 substrate	+0.600	+0.6304	+0.6349	+0.5640	+0.6538	+0.6204	+0.7547	-0.6185	+0.5319
CYP2C9 substrate	- 1.0000	- 1.0000	-1.0000	-1.0000	- 0.6139	- 1.0000	- 0.7915	-0.8283	-0.8006
CYP2D6 substrate	- 0.8031	- 0.8428	-0.8428	-0.7874	+0.4070	- 0.8527	- 0.8348	-0.8434	-0.7235
CYP3A4 inhibition	+0.5057	- 0.8619	-0.8619	+0.6041	- 0.9081	- 0.9310	- 0.9226	-0.7688	-0.8183
CYP2C9 inhibition	+0.6354	- 0.9071	-0.9071	+0.5989	- 0.7281	- 0.9351	- 0.8642	-0.8365	+0.8873
CYP2C1 9 inhibition	- 0.5992	- 0.9025	-0.9025	+0.7387	- 0.7634	- 0.9301	- 0.9010	-0.9060	+0.8995
CYP2D6 inhibition	- 0.9231	- 0.9231	-0.9231	-0.8296	- 0.9411	- 0.9356	- 0.9274	-0.9640	-0.8065

CYP1A2 inhibition	- 0.7709	- 0.9045	-0.9045	-0.7321	- 0.9705	- 0.9113	- 0.9012	-0.7113	+0.7807
CYP inhibitory promiscuity	+0.7391	- 0.6670	-0.6670	-0.5986	- 0.9045	- 0.6965	- 0.9197	-0.9338	+0.8661
UGT catalyzed	+0.6000	+0.6000	+0.8000	+0.6000	+0.6000	+0.7000	0.5000	+0.7000	-0.5000
Carcinogenicity (binary)	- 0.9022	- 0.9714	-0.9714	-0.9571	- 0.9571	- 0.9857	0.9857	-1.0000	-0.9714
Carcinogenicity (trinary)	Non-required 0.7023	Non-required 0.6995	Non-required 0.6995	Non-required 0.6893	Non-required 0.5899	Non-required 0.6809	Non-required 0.5717	Non-required 0.7379	Non-required 0.6706
Eye corrosion	- 0.9843	- 0.9891	-0.9891	-0.9903	- 0.9909	- 0.9896	- 0.9901	-0.9917	-0.9924
Eye irritation	- 0.8041	- 0.9143	-0.9215	+0.6357	- 0.8863	- 0.9294	- 0.9004	-0.6654	-0.6327
Ames mutagenesis	- 0.6300	- 0.6600	-0.6500	-0.6200	- 0.5100	- 0.6000	- 0.6329	-0.7400	-0.5200
Human either-a-go-go inhibition	- 0.5435	+0.7299	+0.7341	+0.7126	+0.8267	+0.7304	+0.8130	+0.7426	+0.7022
Micronuclear	+0.675 9	+0.749 2	+0.749 2	-0.5200	+0.775 9	+0.71 92	- 0.8800	+0.9100	-0.5341
Hepatotoxicity	+0.6000	+0.6500	+0.6750	+0.5750	+0.5250	+0.7250	- 0.7000	+0.7250	+0.6750
Acute Oral Toxicity (c)	III 0.7236	III 0.6904	III 0.6904	III 0.3603	III 0.4016	III 0.6850	I 0.6698	II 0.4858	III 0.6401
Estrogen receptor binding	+0.8483	+0.7993	+0.8064	+0.9135	+0.8022	+0.8101	+0.8063	+0.7663	+ 0.9477

Androge n receptor binding	+0.7342	- 0.8129	-0.6934	+0.5917	+0.7923	+0.6088	+0.7579	+0.7520	+0.7739
Thyroid receptor binding	+0.6674	+0.5588	+0.5642	+0.5320	+0.6124	+0.5388	+0.5791	-0.5637	+0.7536
Glucocor ticoid receptor binding	+0.7895	+0.5657	+0.5967	+0.6102	+0.6084	+0.6481	+0.7618	+0.6422	+0.8291
Aromatas e binding	- 0.6019	- 0.4857	-0.4850	+0.5266	+0.5346	+0.5445	+0.6631	-0.4941	+0.6389
PPAR-γ	+0.6508	+0.7229	+0.7244	+0.8165	+0.7550	+0.6931	+0.8073	+0.8075	+0.8906
Honey bee toxicity	- 0.5000	+0.6324	+0.6324	+0.7562	+0.6377	+0.6678	+0.8824	+0.5293	+0.8250
Biodegra dation	- 0.8500	- 0.8500	-0.8250	-0.8500	- 0.8750	- 0.9000	- 0.7250	-0.9000	-0.9250
crustacea aquatic toxicity	- 0.6400	- 0.5549	-0.5400	-0.6800	+0.6800	- 0.6949	+0.5400	-0.5300	-0.6500
Fish aquatic toxicity	- 0.5170	+0.8477	+0.8477	+0.8451	+0.7637	0.8533	+0.9306	+0.9400	+0.9913
Water solubility	-2.649	-2.649	-2.649	-2.627	-3.374	-2.906	-4.128	-3.077	-4.144
Plasma protein binding	0.907	1.096	1.193	1.301	0.984	1.125	0.778	1.019	0.803
Acute Oral Toxicity	3.332	2.257	2.617	3.364	3.4	2.931	3.703	1.378	2.146
Tetrahym ena pyriformi s	0.746	1.062	1.104	1.33	1.241	1.044	0.656	1.719	1.093

6.3.3- ADMET lab:

9 regression models and 22 classification model is used in this software to improve the performance in this software for prediction of ADME. Large datasets and different representations ensemble techniques and resampling strategy are used to optimize models of unbalanced datasets to get balanced models and to improve its prediction ability.

Table- 6.7: Showing result of ADMETlab:

Properties	**Silymarin**	**Chamuvaritin**	**Neopoucirin**	**Hesperidin**	**Selligueain A**	**Pectolinarin**	**Jujuboside A**	**Terminalin**	**Glabrol**
LogS (solubility)	- 3.622	-3.023	-3.004	-5.663	-5.271	-3.363	- 3.222	-3.584	- 6.149
LogD (distribution coefficient)	0.745	0.974	0.976	1.517	2.264	0.95	2.606	0.524	1.722
LogP (distribution coefficient)	2.363	-1.157	-0.862	5.906	5.126	-0.787	- 2.718	2.654	5.822
Caco2 permeability	- 6.507	-6.622	-6.578	-5.287	-6.51	-6.359	-6.318	-6.564	- 5.134
PPAR-γ	+0.6508	+0.7229	+0.7244	+0.8165	+0.7550	+0.6931	+0.8073	+0.8075	+0.8906
P-gp substrate	0.02	0.09	0.036	0.057	0.148	0.059	0.153	0.093	0.041

HIA (human intestina l Absorpti on)	0.364	0.22	0.22	0.433	0.389	0.242	0.19	0.295	0.61 7
F-20% bioavail ability	0.579	0.579	0.579	0.474	0.491	0.59	0.254	0.493	0.54 2
F-30% bioavaia bility	0.301	0.082	0.095	0.334	0.351	0.206	0.262	0.445	0.40 1
PPB (Plasma protein binding)	84.32 4	73.37	74.917	90.935	70.656	75.677	57.672	77.209	89.6 33
BBB (Blood brain barrier)	0.335	0.178	0.183	0.792	0.595	0.044	0.024	0.908	0.81 3
VD (Volume distributi on)	- 0.663	-0.98	-0.831	-0.534	-0.533	-0.886	-0.371	-0.781	- 0.03 6
CYP1A 2-inhibitor	0.073	0.034	0.041	0.294	0.101	0.127	0.073	0.683	0.35 6
CYP1A 2-substrate	0.469	0.474	0.408	0.302	0.362	0.45	0.32	0.368	0.4
CYP3A 4-inhibitor	0.806	0.069	0.25	0.613	0.659	0.525	0.731	0.042	0.42

CYP3A 4-substrate	0.494	0.069	0.51	0.565	0.556	0.5	0.508	0.266	0.574
CYP2C9-inhibitor	0.166	0.048	74.917	0.559	70.656	0.244	0.532	0.401	0.349
CYP2C9-substrate	0.496	0.305	0.391	0.42	0.401	0.43	0.256	0.566	0.457
CYP2C19-inhibitor	0.314	0.109	0.114	0.743	0.355	0.158	0.169	0.051	0.67
CYP2C19-substrate	0.548	0.488	0.546	0.51	0.404	0.559	0.448	0.471	0.46
CYP2D 6-inhibitor	0.366	0.32	0.288	0.607	0.42	0.368	0.343	0.343	0.46
CYP2D 6-substrate	0.507	0.28	0.378	0.574	0.455	0.438	0.264	0.31	0.369
T-halflife	1.502	1.844	1.794	2.0	2.643	2.174	2.788	2.393	1.978
CL (Clearance)	0.976	0.509	0.57	1.558	0.821	0.637	0.055	0.639	1.868
hERG blockers	0.634	0.58	0.568	0.59	0.68	0.598	0.65	0.574	0.601
HHT (Human hepatotoxicity)	0.412	0.216	0.238	0.3	0.008	0.368	0.0	0.032	0.664

AMES (mutagenicity)	0.142	0.268	0.268	0.474	0.406	0.258	0.244	0.392	0.294
SkinSen (Skin sensitizationn)	0.217	0.207	0.207	0.303	0.309	0.206	0.28	0.291	0.331
LD50 (LD50 of acute toxicity)	3.17	3.112	3.139	2.936	3.277	3.228	3.609	3.189	2.846
DILI (Drug induced liver injury)	0.722	0.756	0.756	0.678	0.716	0.76	0.148	0.868	0.704
FDAMDD (Maximum recommended daily dose)	0.616	0.544	0.514	0.46	0.496	0.388	0.466	0.642	0.502

6.3.4- DruLiTo-

DruLiTo stands for Druglikeness Tool. It is based on set of rules which uses structural properties of compounds for calculation of drug like properties of compounds. It is an open source for virtual screening. It uses Veber rule, MDDR-like rule, Lipinski's rule, Ghose filter, CMC-50 like rule, BBB rule and quantitative estimate of druglikeness (QED) for different calculations.

Table-6.8: Showing result of Druglikeness tool:

Properties	**Silymarin**	**Chamuvaritin**	**Neopoucirin**	**Hesperidin**	**Selligueain A**	**Pectolinarin**	**Jujuboside A**	**Terminalin**	**Glabrol**
MW	482.12	610.19	594.19	452.16	816.21	622.19	1206.6	602	392.2
Logp	0.855	-1.11	-1.029	4.388	0.293	-0.441	1.036	2.486	4.117
Alogp	- 1.848	-3.99	-3.427	2.449	-1.865	-3.996	-5.392	-2.999	3.773
HBA	10	15	14	5	15	15	26	16	4
HBD	5	8	7	3	11	7	14	8	2
TPSA	155.14	234.29	214.06	86.99	259.45	223.29	393.98	267.04	66.76
AMR	132.21	148.91	147.3	145.56	232.01	155.57	280.82	146.94	124.2
nRB	4	7	7	6	4	8	13	0	5

nAtom	57	77	76	58	96	78	178	54	57
nAcidic Group	0	0	0	0	0	0	0	0	0
RC	5	5	5	5	10	5	11	8	3
nRigidB	35	40	39	32	65	40	81	51	26
nArom Ring	3	2	2	4	6	2	0	4	2
nHB	15	23	21	8	26	22	40	24	6
SAlerts	0	0	0	1	0	1	2	3	1

6.3.5- vNN Webserver-

It is a freely available online platform for prediction of ADME properties and to build models on the basis of variable nearest neighbor (vNN) method. It assessess properties like cardiotoxicity, cytotoxicity, drug-drug interactions, mutagenicity, microsomal stability, and drug-induced liver.

Table-6.9: Showing result of vNN Webserver:

Properties	**Silymarin**	**Chamuvaritin**	**Neopoucirin**	**Hesperidin**	**Selligueain A**	**Pectolinarin**	**Jujuboside A**	**Terminalin**	**Glabrol**
DILI	⊘	⊘	⊘	⊘	⊘	⊘	⊘	⊘	⊘
Cytotoxicity	⊘	⊘	⊘	⊘	⊘	⊘	⊘	⊘	⊘
HLM	⊘	⊘	⊘	⊘	⊘	⊘	⊘	⊘	⊘
1A2	⊘	No	No	Yes	No	No	⊘	No	Yes
3A4	yes	No	No	Yes	No	No	⊘	No	No
2D6	No	No	No	⊘	No	No	⊘	No	No
2C9	yes	No	No	Yes	No	No	⊘	⊘	Yes
2C19	No	No	No	Yes	No	No	⊘	No	Yes
BBB	⊘	⊘	⊘	⊘	⊘	⊘	⊘	⊘	⊘

P-gp inhibitor	yes	No	No	⊘	⊘	No	⊘	No	⊘
P-gpsubstrate	⊘	⊘	⊘	⊘	⊘	No	No	⊘	⊘
hER G blocker	⊘	⊘	⊘	No	⊘	⊘	No	⊘	⊘
MMP	⊘	No	No	⊘	⊘	No	⊘	No	⊘
AMES	⊘	No	No	⊘	⊘	No	⊘	No	⊘
MRTD (mg/day)	480	424	300	⊘	4826	298	⊘	⊘	7840

As all 5 different softwares were giving results in different parameters, so 17 common parameters which were there in all 5 softwares were used to compare the results of these softwares collectively, which is given in table 16.

Table 6.10: Showing common parameters from result obtained from above mentioned softwares of ADMET analysis.

Serial no.	**Properties**	**Silymarin**	**Chamuvaritin**	**Neopoucirin**	**Hesperidin**	**Selligueain A**	**Pectolinarin**	**Jujuboside A**	**Terminalin**	**Glabrol**
1	MW	482.44	610.56	594.56	452.5	816.76	622.57	1207.35	602.37	392.49
2	#H-bond acceptors	10	15	14	5	15	15	26	16	4
3	#H-bond donors	5	8	7	3	11	7	14	8	2
4	logP (Distribution coefficient)	2.363	-1.157	-0.862	5.906	5.126	-0.787	- 2.718	2.654	5.822
5	Lipinski #violations	0	3	3	0	3	3	3	3	0

6	TPSA	155.14	234.29	214.06	86.99	259.45	227.2	393.98	282.68	66.76
7	Bioavailability Score	0.55	0.17	0.17	0.55	0.17	0.17	0.17	0.17	0.55
8	LogS (Solubility)	-3.622	-3.023	-3.004	-5.663	-5.271	-3.363	-3.222	-3.584	-6.149
9	LogD (Distribution coefficient)	0.745	0.974	0.976	1.517	2.264	0.95	2.606	0.524	1.722
10	#Rotatable bonds	4	7	7	6	4	8	13	0	5
11	MR	120.55	141.41	139.38	130.98	210.45	148.29	285.51	150.5	116.99
12	logKp (cm/s)	-7.89	-10.12	-10.4	-4.49	-8.16	-9.76	-14.82	-8.62	-4.4
13	Synthetic Accessibility	4.92	6.34	6.27	3.62	7.3	6.63	10	4.19	4.15

14	PAINS #alerts	0	0	0	0	0	0	0	1	0
15	Brenk #alerts	0	0	0	0	0	0	2	4	1
16	PPAR-γ binding	+0.6508	+0.7229	+0.7244	+0.8165	+0.7550	+0.6931	+0.8073	+0.8075	+0.8906
17	P-glycoprotein inhibito r	+0.9511	-0.9166	- 0.8489	+0.7498	+0.7279	- 0.6881	+0.7461	- 0.6070	+0.7841

Optimal range of values of different parameters considered for this in silico ADME analysis is give in the form of table as follows:

Table 6.11: Showing normal value range of different parameters used in ADMET analysis

S. N.	Parameter	Value Range	Reference
1	MW	<500	Bowers K. J. et al 2006
2	#H-bond acceptors	<10	Bowers K. J. et al 2006
3	#H-bond donors	<5	Bowers K. J. et al 2006
4	LogP (Distribution constant)	<5	Bowers K. J. et al 2006
5	TPSA	20 – 130	Sivashanmugam, M. et al, 2019
6	Bioavailability Score	0 – 1	Chen X. et al 2020
7	Log S (Solubility)	>0 – (-4)	Mishra, S. et al 2019
8	Log D (Distribution coefficient)	0 – 3	Price, G. et al 2021
9	#Rotatable bonds	Should be <10	Sorkun, M. C. et al 2019
10	Molar Refractivity	40 – 130	S Bharate, S. et al 2016
11	log Kp (cm/s)	-1.2 – 1.32	Jagannathan, R., 2019
12	Synthetic Accessibility	1 – 10	Ghose, A. K. et al, 1999
13	PAINS #alerts	No. of false positives, value should be less	Chen, C. P. et al, 2018

14	Brenk #alerts	Filter to remove toxic, chemically reactive and metaolically unstable compounds, value should be more	Ertl, P. et al, 2009
15	PPAR-γ binding	Dock score Value should be more	Baell, J. B. et al , 2010
16	P-glycoprotein inhibitor	Value should be more	Daina, A. et al, 2017

As given in table 6, compound silymarin, hesperidin and glabrol are obeying Lipinski rule, which says that Molecular weight (MW<500), H bond acceptor < 10, H bond donor < 5 and Log p < 5, if a drug obeys all these rules then it can be considered as lead compound for drug development. Then other parameters like Total polar surface area (TPSA, which tells about polarity of compound) should be between 20-130. Hesperidin and glabrol are obeying this condition although silymarin is going slightly high, rest of the compounds are going highly out of range. Then bioavailability score is again optimum for silymarin, hesperidin and glabrol only, rest of the compounds are having lesser one. Log S, i.e. watersolubility should be greater than -4, which is being followed by all the compounds. Next parameter is log D, which is a log of partition of a chemical compound between the lipid and aqueous phases i.e. Log D tells that compound would be more hydrophilic or lipophilic. More negative Log D, less lipophillic compound so poor membrane permeability, so for better membrane permeability, value of log D should be less –ve, which is observed for Hesperidin, Selligueain A, Jujuboside A and Glabrol. Then, another parameter is rotatable bond, which is measure of molecular flexibility and is important in assessing oral bioavailability of drug; hence it should be less than 10 for good bioavailability of drug, which is observed for compounds except Jujuboside A. Then molar refractivity (MR), which is molar volume corrected by refractive index. It represents size and polarizability of a fragment or molecule. It should be between 40 to 130, which is observed in silymarin, hesperidin and glabrol only. Then next parameter is log Kp (skin permeation), i.e. measure of skin to absorb any compound, more –ve value of log Kp, less would be the skin permeation, again molecule silymarin, hesperidin and glabrol are best among others asthey have less negative values so more would be the skin permeation of drug. Synthetic accessibility means how much it would be easy to synthesize a drug. If the value is 1 then it would be very

easy to synthesize a drug whereas if the value is 10 then it would be very difficult to synthesize a drug. For this also, again the value of silymarin, hesperidin and glabrol is best among other compound as their value is closer to 1. PAINS (PAN Assay interference compound) Alerts, tells about the presence of particular fragment in drug which will always show positive result, no matter which target is being taken, that means it shows false positive result. It is 0 in all molecules except molecule 8. BRENK Alerts shows thepresence of any particular fragment in drug which could be toxic, reactive and unstable etc., its value is also found 0 for all molecules except 7, 8 and 9. That means all molecules except 7, 8 and 9 do not have any toxic, reactive and unstable fragment. Then, another parameter, PPAR-γ binding, that means ability to bind with PPAR-γ receptor, is also good for 4, 7, 8 and 9. Then, in last P-glycoprotein, which excretesxenobiotics (drug or toxin) outside the body and induces multi drug resistance. Hence cancer cells usually increase number of p-glycoprotein for developing multi drug resistance. So, a good drug should be p- glycoprotein inhibitor. So, value of p- glycoprotein inhibitor should be more then it will be a good drug, its value is found highest for molecule 1 in comparision to others.

6.4- Molecular dynamics-

Although Molecular docking has revealed details of protein-ligand binding, but to assess smallest discrepancy, molecular dynamics simulation studies are required. It tells ultimate details regarding motion of individual atom, as a function of time. Molecular dynamics study was performed in DESMOND 2022.1 software package. Best 3 molecules obtained from ADME analysis were explored to see their atomic detail in solvent system. X-ray ligand of PPAR-γ was chosen as a reference compound. The best confirmation based on RMSD, binding energy and H-bond interaction were taken for molecular dynamics study. The simulation of 200 ns was performed to establish the stability of protein-ligand complex, and further investigation of simulation was done. Structure of PPAR-γ was optimized and subjected to molecular dynamics study in simulated physiological conditions, to study its dynamic behavior, using Newtonian equation. In simulation system, orthorhombic box was calculated, solvated with water molecules i.e. explicit single point charge (SPC), neutralized by addition of Na^+ ions. Subsequently, energy of this solvated system was minimized and OPLS 2005, forcefield was used to restrain the position. After that, 200 nano seconds of molecular dynamics run was done using NVT and NPT ensembles which was further followed by unrestrained production run of 200 ns, with interval of 1.0 ps. While production runs, temperature was kept at 300K and

pressure at 1 atmosphere. The root mean square deviation (RMSD) for protein backbone and root mean square fluctuation (RMSF) and radius of gyration were plotted for ligands to see how much the structure converged is to equilibrium.

6.4.1- Analysis of RMSD of PPAR-γ backbone for molecular dynamics trajectory with different ligand complex:

RMSD value tells about divergence of position of different atoms of protein w.r.t. reference. Smaller RMSD indicates greater similarity in structures. In fig.23, RMSD plot is generated to elaborate structural stability and dynamic behavior of PPAR-γ. It is shown in fig. 23 that RMSD of all the ligands is within normal range, i.e. 2-3. However, there is steep increase in backbone deviation of PPAR-γ due to silymarin, up to 1.5 Å during 0 to 100 ns, while backbone deviation due to hesperidin increases up to 1Å, whereas RMSD of PPAR-γ backbone coincided for both glabrol and x-ray ligand. However, all the complexes of ligands and protein attained equilibrium after 130 subsequently, it remained stable throughout the simulation time for up to 200 ns. This indicates presence of stably evolved system during the simulation process.

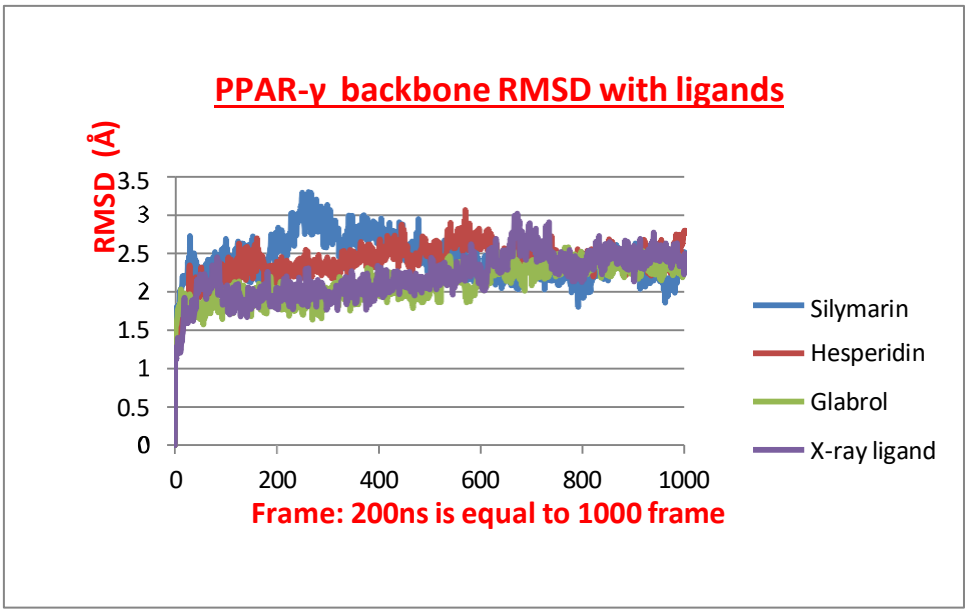


Figure 6.4: Showing RMSD of PPAR-γ backbone w.r.t. Silymarin, Hesperidin, Glabrol and X-ray ligand of PPAR-γ

6.4.2- Analysis of RMSD of different molecule complexes with respect to PPAR-γ from molecular dynamics trajectory:

In trajectory analysis, in fig.24, the RMSD of x-ray ligand was within normal range i.e. between 1 to 3, while RMSD of hesperidin is in close proximity with x-ray ligand, but silymarin shows increase in RMSD around 120 ns. However, steep increase was observed in RMSD of glabrolfrom 0 to 100 ns. However, system got converged at 150 ns and sustained throughout the simulation run with minimum conformational changes till 200ns.So, the RMSD was found to be near constant after half of the MDS run and no much structural changes were observed in the protein structure during the binding of the molecules into the active site. These findings strongly reinforce these molecules were stable binder for PPAR-γprotein.

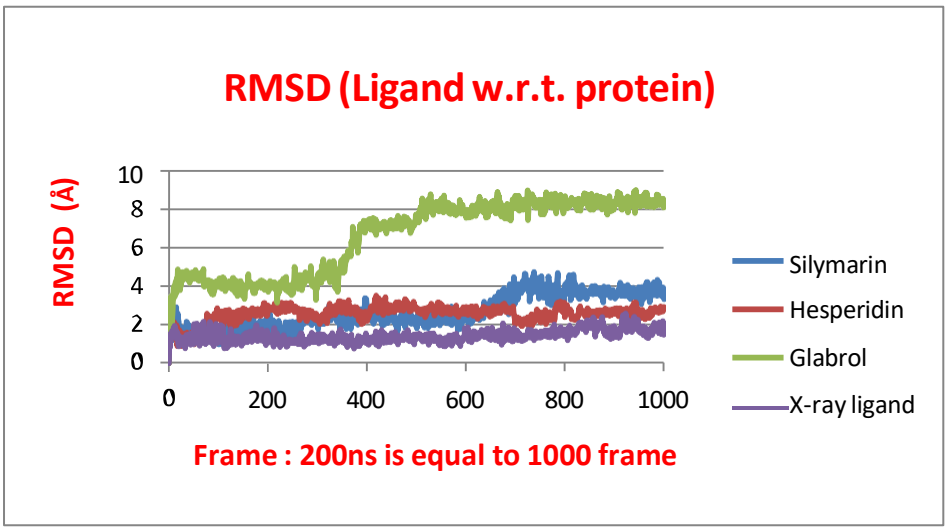


Figure 6.5: Showing RMSD of PPAR-γ (Lig w.r.t. prot.) for all 4 ligands i.e. Silymarin, Hesperidin, Glabrol and X-ray ligand of PPAR-γ

6.4.3- Analysis of RMSF of protein backbone:

To understand the dynamics of simulated complex in more detail, time averaged RMSF was computed for protein bound with silymarin, hesperidin, and glabrol and x-ray ligand at 300K. The RMSF evaluates average fluctuation of residues of protein over time with a reference position i.e. fluctuations along the protein chain before and after binding with different ligands. Higher RMSF value indicates more flexibility. Changes in RMSF of protein backbone w.r.t. ligands werefound fluctuating very much but in synchronization with each other as given in fig.25. RMSF for all the ligands increased suddenly at residue 34 from 32, but increase was just double for silymarin, then again decrease in RMSF was observed for all the ligands at residue

40. Again, significant increase in RMSF was observed near residue 61 for all the ligands, then starts decreasing and then found increasing near residue 69 then againdecreasing. RMSF dropped significantly near residue 74 and then increased at residue 107 for all the ligands. Further again decrease in RMSF was observed for all ligands near 118 residue which got increased near 219 different ligands in different amount, which further found to decrease again.

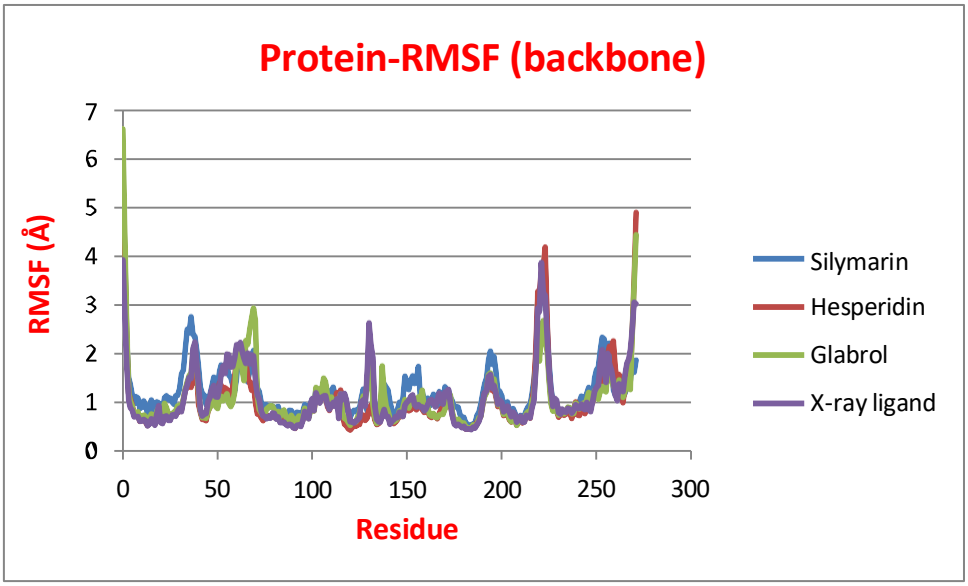


Figure 6.6: Showing RMSF of protein backbone

6.4.4- Analysis of radius of gyration of protein:

Radius of gyration deals with the distribution of residues of protein around its axis, determining protein structure compactnessduring MDS.It tells about the shape of the molecules during the simulation. In fig. 26, radius of gyration of the proteins were coinciding for almost all the ligands showing similarity in distribution of atoms around its axis. In all the complexes, protein was found to be stable, i.e. no much structural changes during the MDS. The overall compactness of the protein in presence of the molecules was varied from 19.1 to 19.8 Å which seems stable.

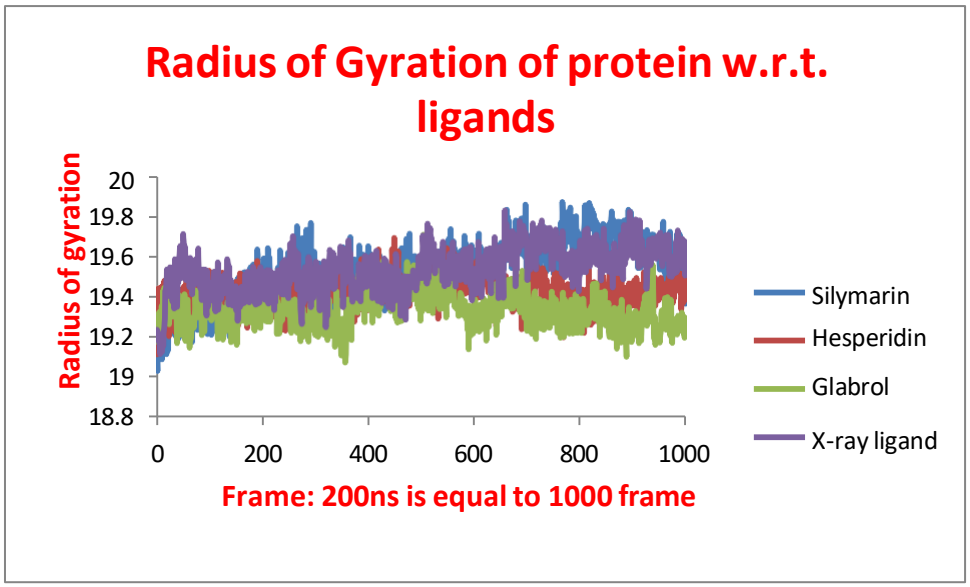


Figure 6.7: Showing radius of gyration of protein w.r.t. ligands

6.4.5- Ligand- Protein binding interactions in Desmond:

The contacts of different residues of silymarin and PPAR- γ were seen in MDS studies (as shown in figure 27. Stronghydrophobic interactions were found withresidues Phe264 (45%), His266 (30%), Phe287 (35%) and Met364 (25%) during the MDS run. Strong H-bonding interactions with Silymarin were observed with residues Lys265 (65%), Ile281 (45%), Cys285 (22%) and Lys367 (20%). Strong water bridging was also observed with the residue Arg288 (70%). Low water bridgingwas also involved with the residues Lys263, Phe287, Glu291, Ser342, Glu343, etc. However, low (<20%) hydrogenbonding and hydrophobic interactions were also observed during MDS run (as depicted in Figure 27a). More than one interaction was also observed by the different residues during the simulation (Figure 27a). The % of the interactions means, they are observed the % time from the whole MDS run. High fraction of the interactions like H-bonding, Hydrophobic and Water bridging) during MDS, means it had more contribution in making the molecule stable into the active site and consistently had contact withthe protein via different interactions throughout the simulation (Figure 27b).

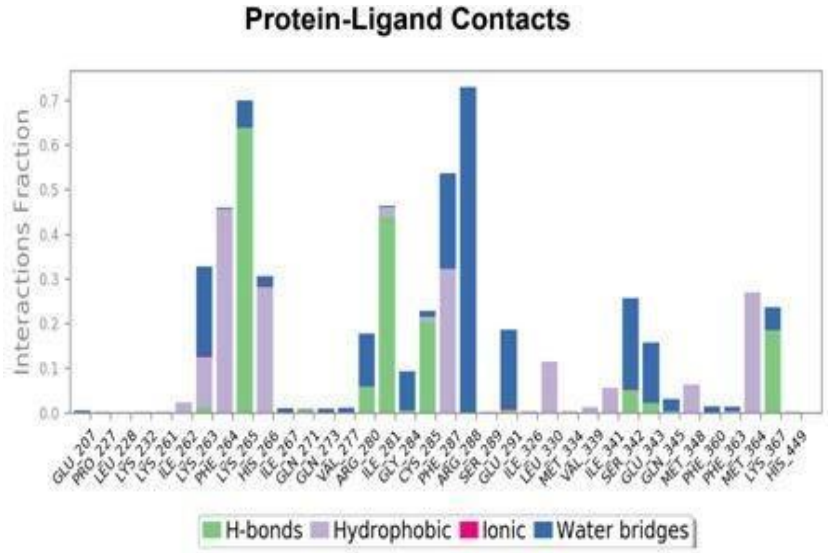


Figure 6.8(a): Shows bar chart for PPAR-γ-silymarin contacts observed during the MDS

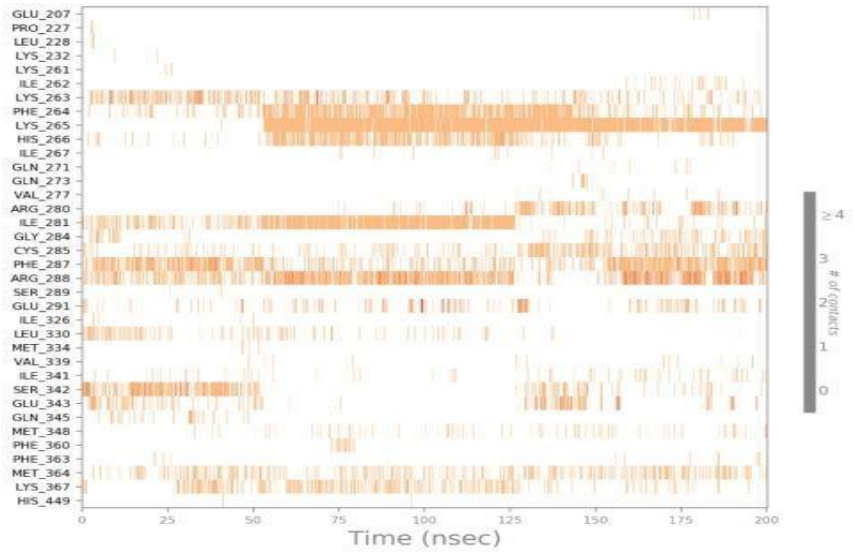


Figure: 6.8(b) Shows Plot for PPAR-γ-silymarin interactions in each time frame (in ns scale)

Interactions of glabrol with PPAR- γ (Figure 28), exhibits different kind of interactions like H-bonding, hydrophobic and water bridging. The major H-bonding interaction wasIle281 (23%), Ser342 (50%), Lys367 (33%) and Glu343 (~20%). The hydrophobic interactions with the residues Tyr327 (30%), Ile341 (25%), Phe363 (20%), Phe264 (23%) were observed. The strong

water bridging with Ser289 (30%)and Tyr473 (30%) was also observed (Figure 28). In addition, other interactions were also observed (Figure 28) but had less fraction of contribution during the MDS.

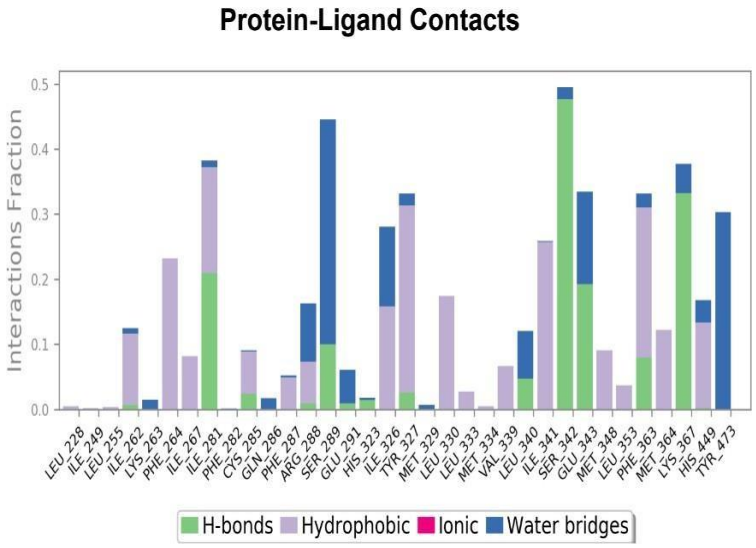


Figure: 6.9 (a) The bar chart shows for PPAR-γ-glabrol contacts observed during the MDS

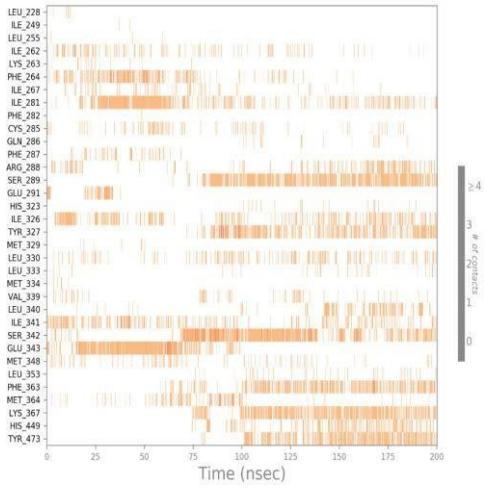


Figure 6.9 (b): Shows plot for PPAR-γ-glabrol interactions in each time frame (in ns scale).

On the other hand, Hesperidin was also participating in the different types of the interactions with the PPAR-gamma. The Strong H-bonding was found with the residues Gln272 (100%), Gln273 (50%), Arg280 (65 %) and Leu270 (40%). The hydrophobic interactions were observed with the Phe264 (50%), Met329 (25%), Leu330 (30%) and Ile341 (40%). The Hesperidin was also interacted via water bridging with the residues Gln271 (50%), Gly284 (65%), Ser342 (50%), Glu343 (80%) as per given the figure 25. Some of the interactions were also observed with the residues but their occurrence found less during the simulation (Figure 29). In case of the X-ray ligand of the PPAR-gamma (PDB ID: 3V9T), mainly hydrophobic interactions [Leu330 (45%), Ile341 (40%) Met334 (30%)] and water bridging [with Arg280 (95%), Leu340 (40%) and Ser342 (100%)] with the residues as depicted in figure 30. It was observed from the Figure 27-30, all the molecules had good binding interactions with the active site residues which were found to be important for the binding with the PPAR-gamma. However, these molecules also exhibited the high-water bridging with the residues as mentioned in the figure 27-30. Thewater bridging has role in the MDS as it mediates interactions between ligands and protein residues via H-bonding. It can affect the stability and binding affinity of the ligand with protein, thereby influencing the protein-ligandbinding kinetics. Here water bridging was observed in protein-ligand binding via H- bonding (wateract as both donor and acceptor (Figure 29a-30a), means protein get stabilized due to this event in each complexes and reflected in the RMSD, RMSF and radiation of gyration plots (as discussed above).

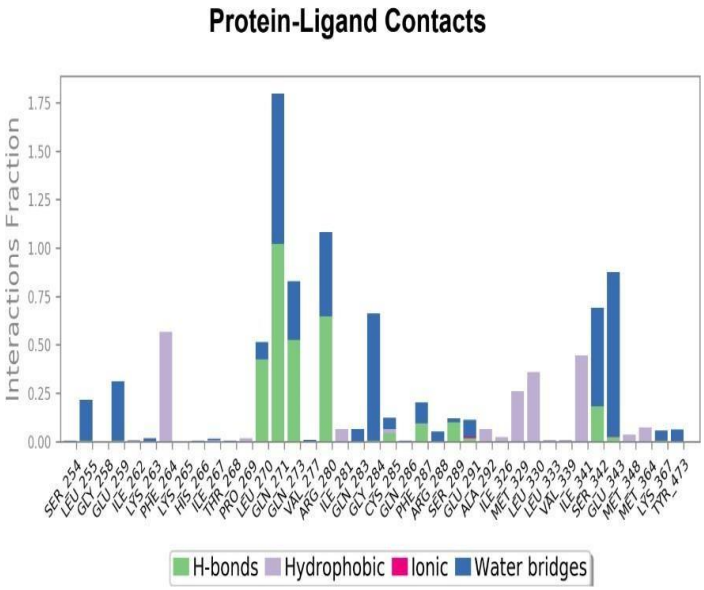


Figure 6.10 (a): Bar chart for PPAR-γ- Hesperidin contacts observed during the MDS

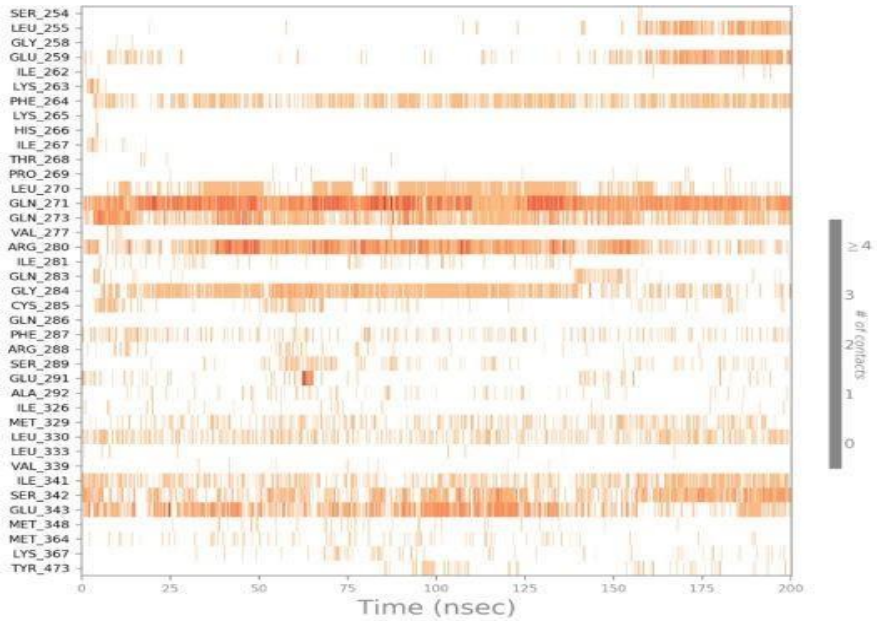


Figure 6.10 (b): Plot for PPAR-γ- Hesperidin interactions in each time frame (in ns scale)

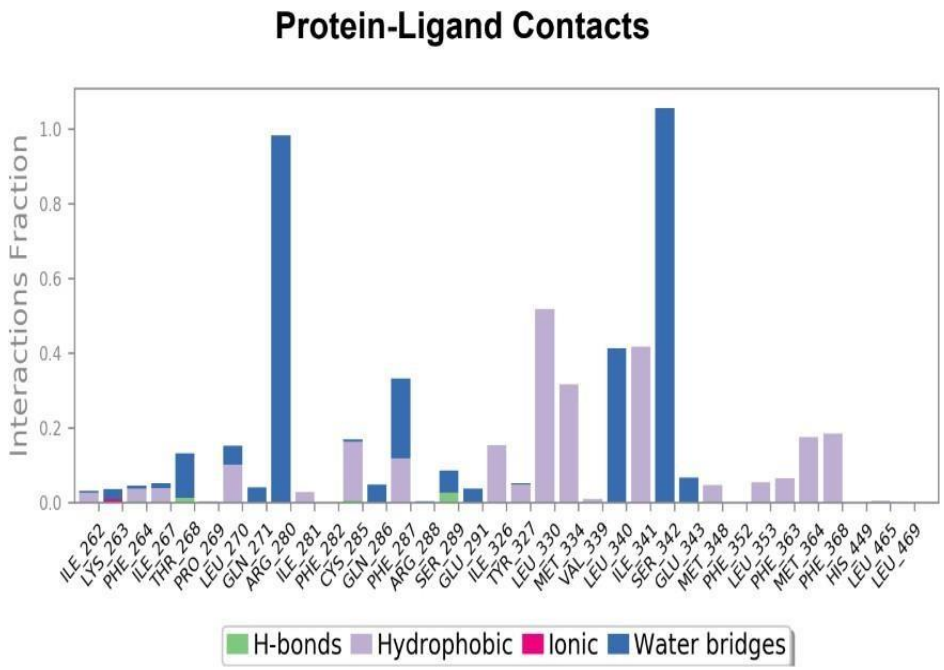


Figure 6.11 (a): Bar chart for PPAR-γ-X-ray ligand (PDB ID: 3V9T) contacts observed during the MDS.

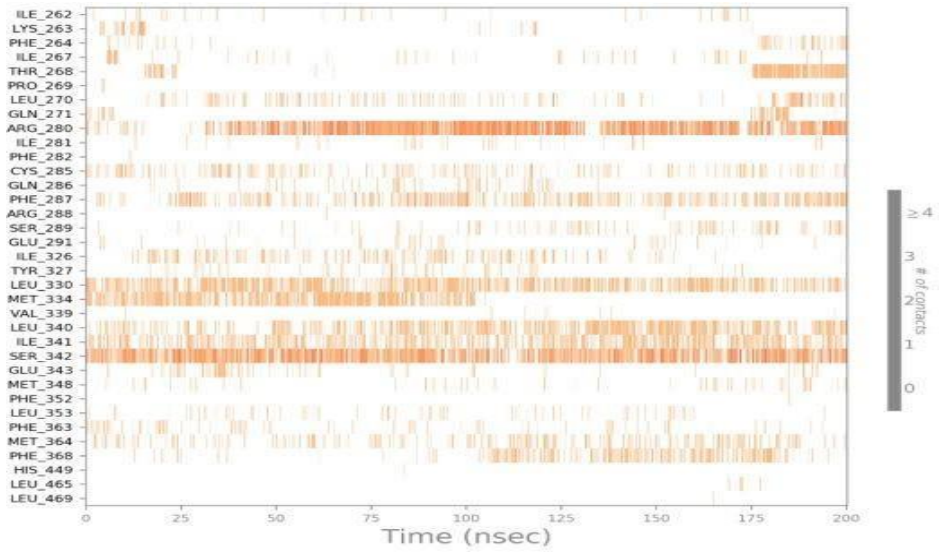


Figure 6.11 (b): Plot for PPAR-γ-X-ray ligand interactions in each time frame (in ns scale)

Moreover, the binding free energy for each protein ligand complex (Silymarin, Hesperidin and Glabrol) was also calculated in Prime tool of Schrodinger package from last 100 ns. It was found that Hesperidinwas having high binding free energy (-85.958 kcal/mol) as compared to co- crystallized ligand (-82.951 kcal/mol). However, other molecules Silymarinand Glabrol were having less binding free energy (-72.036 kcal/mol and 71.054 kcal/mol, respectively) as compared to crystallized ligand. The Overall MDS resultsreflected that Hesperidinwas found good molecules against PPAR-gamma protein asanagonist. This could be potential lead against PPAR-gamma but need to be proven experimentally.

The overall results of these studies, found that Hesperidin had high binding potential against PPAR-gamma as compared to Silymarin and Glabrol. However, all the compounds were found stable during the RMSD, RMSF and radiation of gyration studies. These studies firsttime reported that the compounds against PPAR-gamma as an agonist to understand their molecular mechanism and to depict the important sight points for binding.

Among all these 3 compounds i.e. hesperidin, silymarin and glabrol, we chose hesperidin and silymarin for invitro studies, glabrol was good but it was very expensive so we didn't consider it for further studies.

6.5- Invitro studies-

In whole invitro study, MDA MB-231 cell line is used for performing different type of assays. Triple- Negative Breast Cancer (TNBC) Model includes MDA-MB-231 cell line as it is a human breast cancer cell line derived from a patient with TNBC**,** meaning it lacks: Estrogen receptors (ER), Progesterone receptors (PR) and HER2**,** thus it closely mimicks TNBC study model. They are highly invasive and metastatic, mimicking the aggressive nature of TNBC in patients. They display a mesenchymal morphology (spindle-shaped, loosely attached). High expression of epithelial-mesenchymal transition (EMT) markers like vimentin and low E-cadherin is found in this cell line. Due to their aggressive behavior and drug resistance, they are ideal for: testing novel chemotherapies, evaluating invasion, metastasis, and EMT. It harbors mutations commonly found in TNBC: KRAS and TP53 mutations (TP53 is mutated in ~80% of TNBCs), lacks PIK3CA mutation, consistent with basal-like TNBC. TNBC is aggressive and lacks targeted therapies making MDA-MB-231 a relevant in vitro model to test cytotoxic and oxidative stress-based therapeutic strategies. MDA-MB-231 is: Fast-growing and metabolically active, giving reliable MTT signals. It is resistant to many drugs, mimicking chemoresistance in TNBC. It's ideal for evaluating anti-proliferative effects of candidate drugs or natural compounds.

6.5.1- DPPH free radical scavenging assay-

DPPH assay tells about the antioxidant potential of test compounds. Free rasdical scavenging activity of Silymarin and Hesperidin was measured using protocol explained by Sharma et al, 2009. Both Silymarin and Hesperidin showed good antioxidant activity in comparision to standard Ascorbic acid. The DPPH percent inhibition activity of Silymarin was found as 55.42±1.09, 63.05±2.42, 78.20±2.62, 88.78±0.36, 102.51±2.78 with its increasing concentration as 10µg/ml, 20µg/ml, 40µg/ml, 80µg/ml, 160µg/ml and IC_{50} as 30.35991. Whereas, DPPH percent inhibition activity of Hesperidin was found to be as 37.16±0.76, 44.76±0.28, 48.31±0.60, 56.87±1.25, 71.21±3.79 with its increasing concentration as 10µg/ml, 20µg/ml, 40µg/ml, 80µg/ml, 160µg/ml and IC_{50} as 53.171041. As a standard, DPPH percent inhibition activity of Ascorbic acid was found as 45.56±1.57, 55.70±3.15, 73.63±0.64, 81.51±4.80, 91.81±8.23 with its increasing concentration as 10µg/ml, 20µg/ml, 40µg/ml, 80µg/ml, 160µg/ml and IC_{50} as 2.8319623. This assay was done in triplicates.

Table 6.12: Showing antioxidant activity of Silymarin and Hesperidin at different

concentrations, along with standard Ascorbic acid.

Concentration (µg/ml)	Antioxidant activity of Silymarin	Antioxidant activity of Hesperidin	Antioxidant activity of Ascorbic acid
10	55.42±1.09	37.16±0.76	45.56±1.57
20	63.05±2.42	44.76±0.28	55.70±3.15
40	78.20±2.62	48.31±0.60	73.63±0.64
80	88.78±0.36	56.87±1.25	81.51±4.80
160	102.51±2.78	71.21±3.79	91.81±8.23

Data is represented as mean±SE

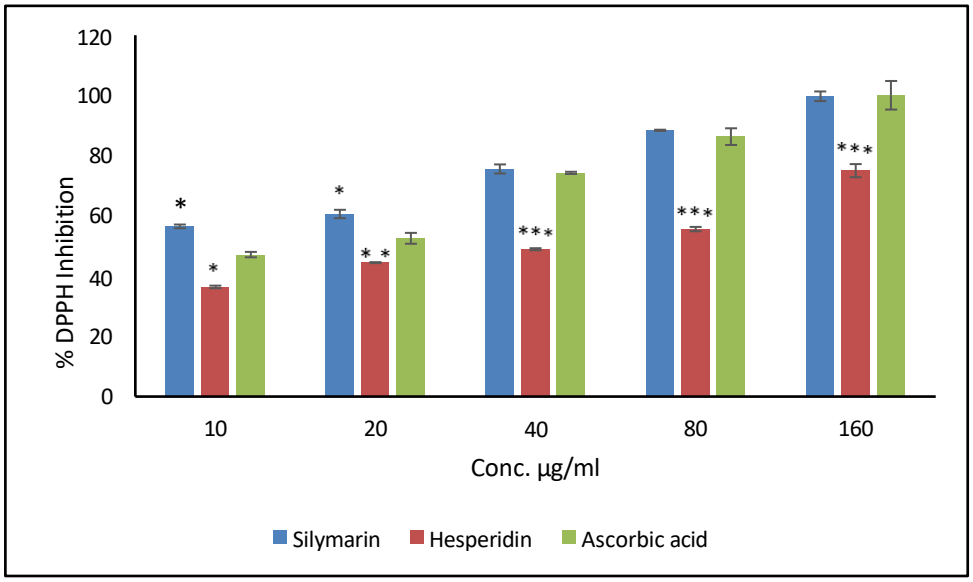


Figure 6.12: Free radical scavenging activity of Silymarin, Hesperidin and Ascorbic acid. The data is given as mean ± SEM of 3 independent readings. Values are means ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001**

IC50 of Silymarin was 30.35991, whereas IC50 of Hesperidin was 53.171041 and IC50 of Acsorbic acid is 2.8319623. As IC50 of Silymarin is lower than Hesperidin, so Silymarin will be considered of higher antioxidant potential than Hesperidin. As the values of % DPPH inhibition by both compounds are less than 0.05 level of significance, so, results are statistically significant.

6.5.2- Cell viability assay on MDA-MB 231 cell line-

Cell viability of triple negative breast cancer cell line, MDA-MB 231 was assessed using increasing concentrations of test compounds Silymarin, Hesperidin and standard drug compound Doxorubicin (used for treatment of triple negative breast cancer). This assay was done to know that how many cancer cells will remain alive after treatment with test compounds. So, the result of % cell viability obtained after this assay for Silymarin was 71.00±1.90, 55.46±3.43, 40.91±3.31, 31.11±0.93 at increasing concentration as 3µM, 6µM, 12µM and 24µM. Whereas, the value of % cell viability obtained for Hesperidin was 87.71±1.65, 76.43±0.69, 63.30±2.11, 57.03±1.20 at increasing concentration as 3µM, 6µM, 12µM and 24µM. As a standard drug, the value of % cell viability obtained for Doxorubicin was 34.94±2.71, 24.53±2.86, 16.92±2.62, 11.67±3.28 at increasing concentration as 3µM, 6µM, 12µM and 24µM.

Table 6.13: Showing % cell viability of test compounds at increasing concentrations

Concentration	**% cell viability of**	**% cell viability of**	**% cell viability of**
(µM)	**Silymarin**	**Hesperidin**	**Doxorubicin**
3	71.00±1.90	87.71±1.65	34.94±2.71
6	55.46±3.43	76.43±0.69	24.53±2.86
12	40.91±3.31	63.30±2.11	16.92±2.62
24	31.11±0.93	57.03±1.20	11.67±3.28

Data is represented as mean±SEM

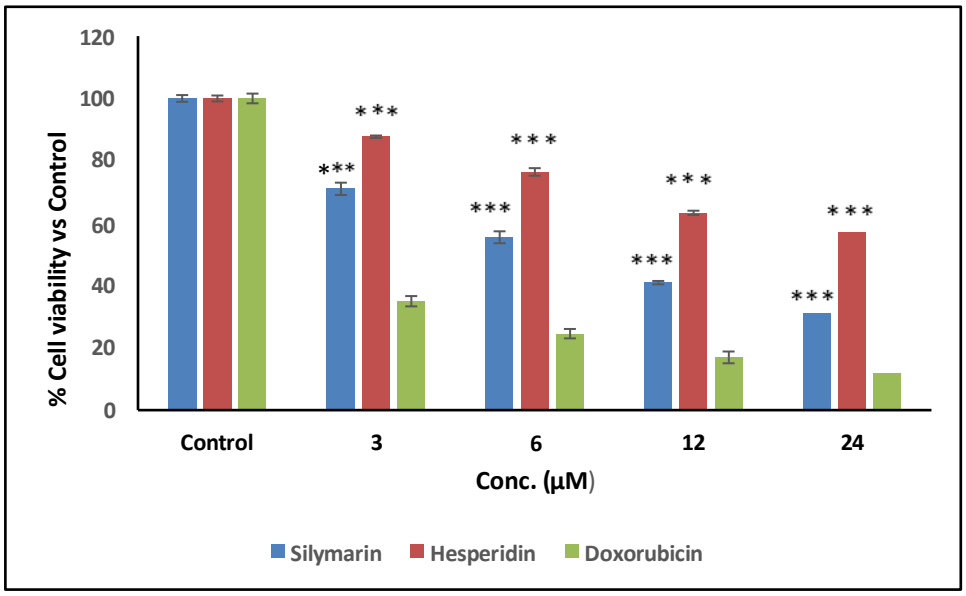


Figure 6.13: Showing MTT analysis of Silymarin, Hesperidin and Doxorubicin w.r.t. Control on MDA-MB-231 cells. The data is given as mean ± SEM of 3 independent readings. Values are means ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001**

IC_{50} of silymarin was found to be 2.47 µM, while IC_{50} of hesperidin and doxorubicin was found to be 26.85 µM and 1.11 µM respectively. Thus, Silymarin is although less potent than doxorubicin but still more effective than hesperidin as it shows better cytotoxic activity against cancer cell. Hesperidin showed weaker cytotoxic activity or anticancer activity than Silymarin and doxorubicin. As the p values of MTT assay for both compounds are less than 0.001 level of sigificance, so, results are statistically significant.

6.5.3- Cell viability assay on L929 cell line-

Cell viaility assay was also performed on normal L929 which is an adherent type of mouse fibroblast cell line, to assess that whether the test compounds kill normal cells too or not. The result of cell viability assay when performed with Silymarin, Hesperidin and doxorubicin on L929 cell line, following result was obtained. IC_{50} of Silymarin was found to be 17.45 µM, IC_{50} of Hesperidin was found to be 26.01 µM, IC_{50} of doxorubicin was found to be 10.33 µM.

Table 6.14: Showing values of % cell viability of test compounds Silymarin, Hesperidin and standard compound after treatment of L929 cell line

Concentration (µM)	% cell viability of Silymarin	% cell viability of Hesperidin	% cell viability of standard
3	93.49±0.94	93.78±0.25	85.94±1.46
6	80.11±0.40	83.93±1.34	76.17±1.67
12	70.18±1.35	75.91±2.34	66.21±2.45
24	57.40±1.58	60.80±2.55	43.50±1.24

Data is represented as mean±SEM

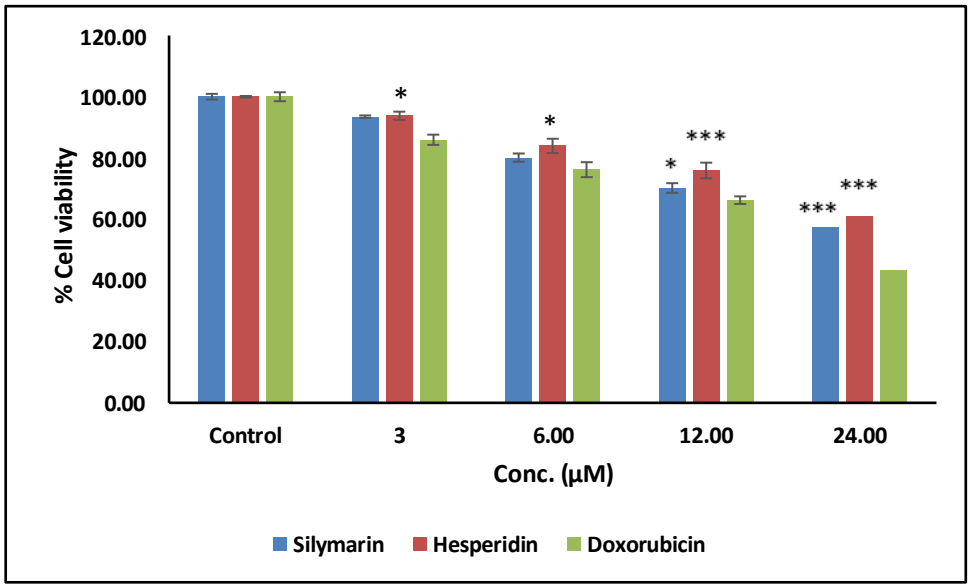


Figure 6.14: Showing MTT analysis of Silymarin, Hesperidin and Doxorubicin on L929 Cells. The data is given as mean ± SEM of 3 independent readings. Values are means ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001**

IC_{50} of Silymarin was found to be 17.45 μM, IC_{50} of Hesperidin was found to be 26.01 μM, IC_{50} of doxorubicin was found to be 10.33 μM. Here, doxorubicin being standard compound is most toxic to L929 cells. As a chemotherapeutic agent, doxorubicin causes significant cell death even at low concentration. Its potency here reflects strong toxicity to normal cells. However, IC50 of Silymarin is more than doxorubicin, so it is moderately cytotoxic or less toxic than doxorubicin indicating better biocompatibility. Whereas, Hesperidin having highest IC50 is least cytotoxic to L929 cells and is most biocompatible compound and least toxic to normal fibroblast cells. As the values of MTT assay by both compounds are less than 0.05 level of sigificance, so, results are statistically significant. L929 cells are used in MTT assay as they are standardized and well characterized murine fibroblast cell line, suitable for cytotoxicity testing. As these cells are normal, so to see whether compounds are toxic to normal cells or not, this cell line was used.

6.5.4-Transcription factor binding assay-

Transcription factor binding assay was performed using 96 well ELISA plate with the help of PPAR-γ transcription factor assay kit item no. 10006855 on MDA-MB 231 cells. This assay was accomplished by attaching PPRE containing double stranded DNA sequence onto the bottom of 96 well plate. Then, activated PPAR-γ present in cell lysate binds onto the PPRE sequence in immobilized DNA. This bound PPAR-γ was identified by binding with primary antibody which further is detected by secondary antibody. After which, absorbance was taken at 450 nm. Dose dependent, non-linear increment in % PPAR-γ agonistic activity of natural compounds Silymarin and Hesperidin was observed and was compared with the synthetic PPAR-γ agonist, pioglitazone as standard. Simultaneously, % PPAR-γ agonistic activity of doxorubicin was also measured. PPAR-γ agonistic activities of these compounds were measured at increasing concentration as 3μM, 6μM, 12μM, 24μM. The result obtained for Silymarin was 107±7.34, 111.66±7.13, 124.66±2.49 and 133.33±4.18 at concentrations 3μM, 6μM, 12μM, 24μM respectively. Whereas for Hesperidin, result obtained was 122±3.26, 129.66±5.24, 136±4.54 and 155.33±9.03 at concentrations 3μM, 6μM, 12μM, and 24μM respectively. In addition to this, % PPAR-γ agonistic activity of synthetic ligand pioglitazone was also measured and the obtained results were as 127.66±3.68, 149.66±6.79, 176±8.60, 191±2.44 at concentrations 3μM, 6μM, 12μM, 24μM respectively. PPAR-γ agonistic activity of standard drug Doxorubicin was also measured and the results found were as 95±2.94,

78±4.89, 72.33±1.24, 67.33±3.85 at concentrations 3µM, 6µM, 12µM, and 24µM respectively.

Table 6.15: Showing % PPAR-γ agonistic activity of different ligands of PPAR-γ at different concentrations

Concentration (µM)	% PPAR-γ agonistic activity of Silymarin	% PPAR-γ agonistic activity of Hesperidin	% PPAR-γ Agonistic activity of ioglitazone	% PPAR-γ agonistic activity of Doxorubicin
3	107±7.34	122±3.26	127.66±3.68	95±2.94
6	111.66±7.13	129.66±5.24	149.66±6.79	78±4.89
12	124.66±2.49	136±4.54	176±8.60	72.33±1.24
24	133.33±4.18	155.33±9.03	191±2.44	67.33±3.85

The data is provided as mean±SEM of 3 independent test

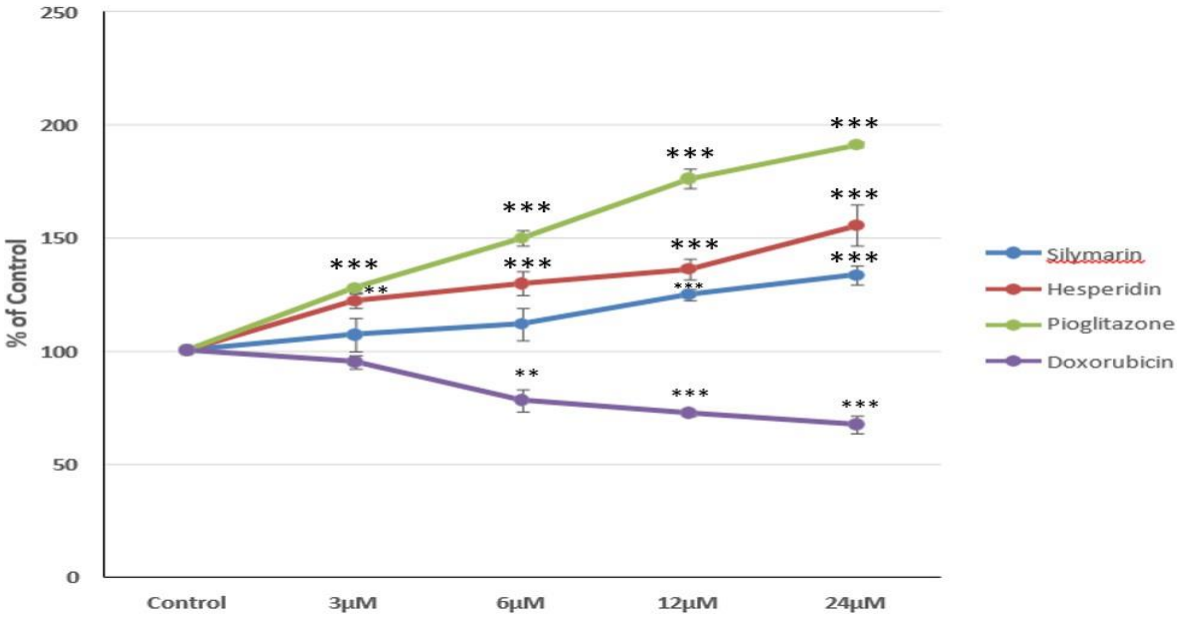


Figure 6.15: Showing % PPAR-γ agonistic activity of different ligands of PPAR-γ atdifferent concentrations, the data is given as mean ± SEM of 3 independent readings. Values are means ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001**

As the values of p for PPAR-γ agonistic activity of different ligands of PPAR-γ at different concentrations are less than 0.01 level of sigificance, so, results are statistically significant. Here, hesperidin is found as better PPAR-γ agonist than silymarin as visible in graph.

6.5.5- Total protein estimation of cells-

Total protein estimation of cells was done by method explained by Lowry et al 1951. Standard curve was made with bovine serum albumin (BSA). It is essential to estimate total protein content before proceeding for any other analysis (Smith et al, 1985) like enzymatic assays, as all enzymes are also protein. So, total protein is estimated to assess the enzyme activity. Specific activity of enzyme is measured in enzymatic analysis as it provides the measurement of purity of enzyme and useful for further analysis. Lowry's method depends upon the presence of aromatic amino acids tyrosine and tryptophan.

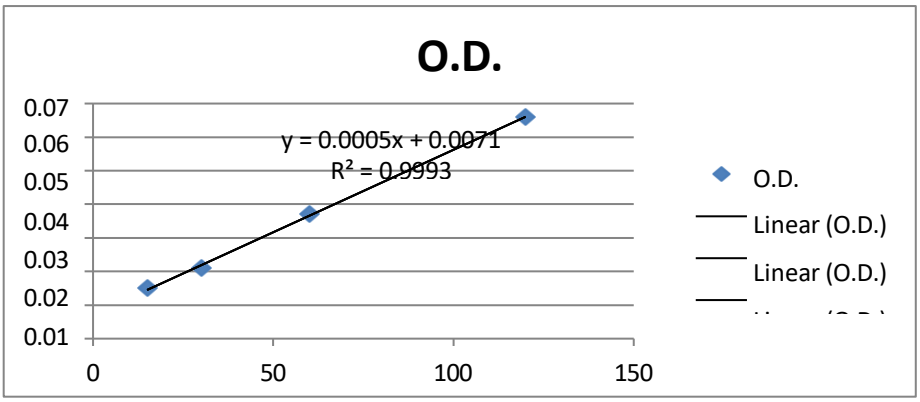


Figure 6.16: Total protein estimation by Lowry's method

6.5.6- Antioxidant enzyme assays-

Antioxidant enzymes like catalase, peroxidase, and glutathione-s-transferase and superoxide dismutase are naturally present in our cells which has action of counterbalancing reactive oxygen species and free radicals generated during various types metabolic reactions. This is basically body's endogenous defence strategy to safeguard our body against different types of diseases like cancer, diabetes etc. So, different enzymatic assays were performed to assess the antioxidant potential of test compounds silymarin and hesperidin inside the living cells that how they affect the activity of these enzymes.

6.5.6.1- Glutathione-S-transferase (GST) activity-

This is a colorimetric assay performed to assess GST activity when cells MDA-MB 231 were treated with natural compounds silymarin, hesperidin and standard drug doxorubicin. The result of GST activity obtained, when cells were treated with these compounds at concentrations 6µM and 12µM respectively, was as 0.067±0.031, 0.033±0.009,

0.024±0.004, 0.079±0.003, 0.077±0.002, 0.385±0.045, 0.402±0.033.

Table 6.16: Showing GST activity in MDA-MB 231 cells when treated with control, doxorubicin, silymarin and hesperidin at concentrations 6µM and 12µM.

Concentration	GST activity
Control	0.067±0.031
Doxorubicin 6 µM	0.033±0.009
Doxorubicin 12 µM	0.024±0.004
Silymarin 6 µM	0.079±0.003
Silymarin 12 µM	0.077±0.002
Hesperidin 6 µM	0.385±0.045
Hesperidin 12 µM	0.402±0.033

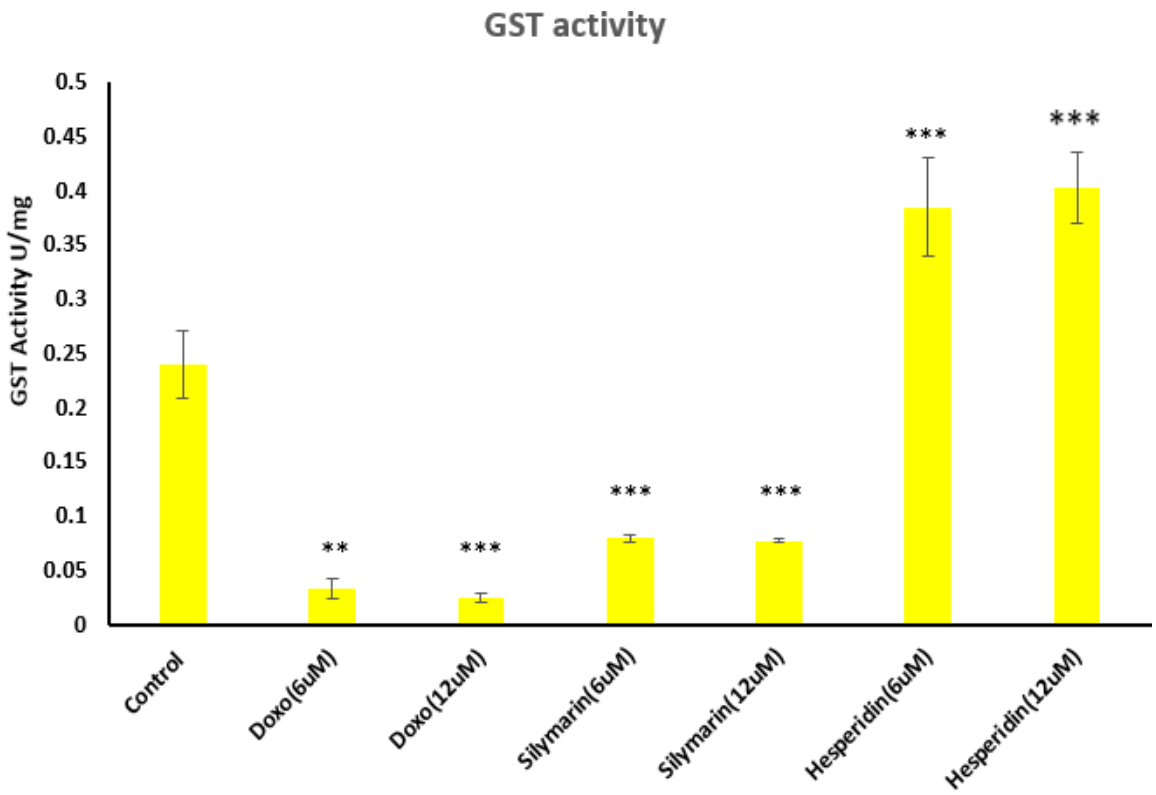


Figure 6.17: Showing effect of silymarin, hesperidin and doxorubicin treatment on GST in MDA-MB 231 cell line. Values are means ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001 when in comparison to control group**

Hesperidin has highest GST activity, shows it has highest antioxidant properties among all

compounds, with highest level of significance. As the values of p for silymarin, hesperidin and doxorubicin treatment in GST assay on MDA-MB 231 cell line are less than 0.01 level of sigificance, so, results are statistically significant. These statistically significant differences indicate that the observed increases in GST activity with hesperidin and silymarin are not due to random chance and reflect real biological effects. Hesperidin's significantly higher GST activity suggests it strongly enhances antioxidant defense mechanism in MDA-MB 231 cells, while doxorubin's significantly lower GST activity confirms its known role in inducing oxidative stress and depleting cellular defense enzymes. MDA-MB-231 is a human breast cancer cell line derived from a patient with TNBC. TNBC is aggressive and lacks targeted therapies making MDA-MB-231 a relevant in vitro model to test cytotoxic and oxidative stress-based therapeutic strategies. TNBC, including MDA-MB-231, typically has elevated ROS (reactive oxygen species) levels. This makes them more sensitive to Pro-oxidant treatments (which induce apoptosis). Antioxidant enzyme activity changes, like: SOD (superoxide dismutase), CAT (catalase), GST (glutathione s transferase). Measuring these enzyme activities helps assess whether a compound causes oxidative stress or modulates redox balance, which is crucial in TNBC pathology.

6.5.6.2- Lipid peroxidation (LPO) activity-

Lipid in our body is usually peroxidized to produce malonyldialdehyde (MDA) by reactive oxygen species produced by different metabolic reactions. The amount of MDA produced was assessed during measuring LPO activity. The result obtained when cells were treated with test compounds control, silymarin, hesperidin and doxorubicin at concentrations 6µM and 12µM respectively, was as 0.006±0.003, 0.095±0.004, 0.091±0.0005, 0.023±0.003, 0.012±0.001, 0.024±0.0005, 0.007±0.002.

Table 6.17: Showing LPO activity in MDA-MB 231 cells when treated with control, doxorubicin, silymarin and hesperidin at concentrations 6µM and 12µM.

Concentration	LPO activity
Control	0.006±0.003
Doxorubicin 6 µM	0.095±0.004
Doxorubicin 12 µM	0.091±0.0005
Silymarin 6 µM	0.023±0.003
Silymarin 12 µM	0.012±0.001
Hesperidin 6 µM	0.024±0.0005
Hesperidin 12 µM	0.007±0.002

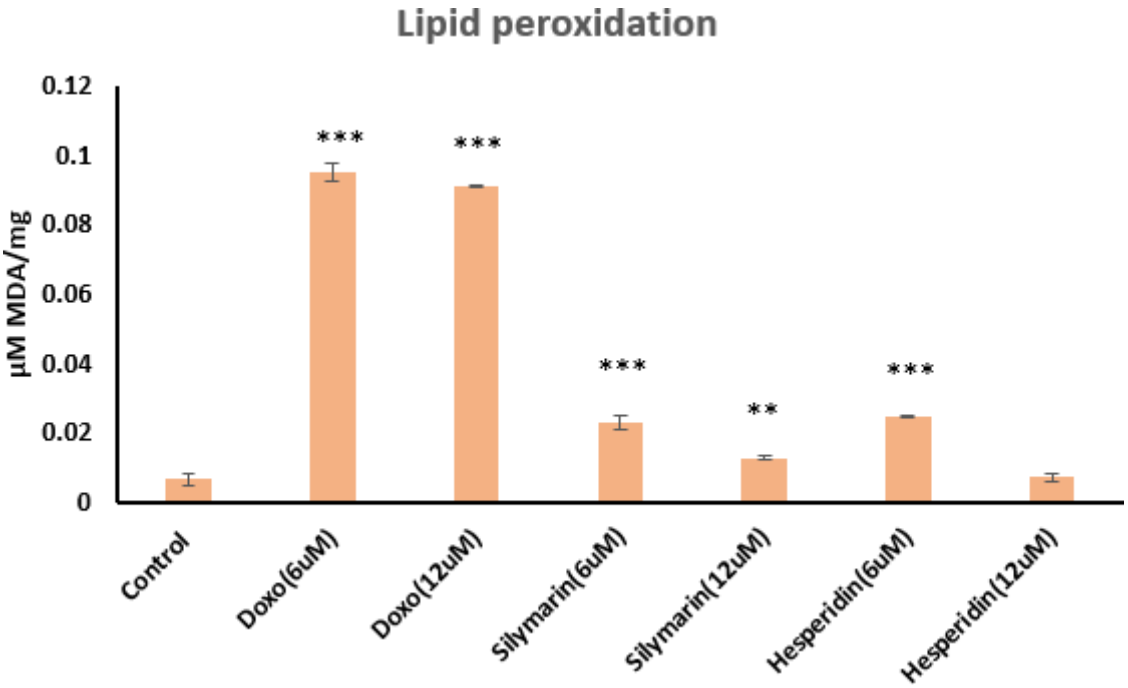


Figure 6.18: Showing result of lipid peroxidation when MDA-MB 231 cells were treated with different natural compounds. Values are means ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001 when in comparison to control group**

Doxorubicin, a chemotherapeutic agent is well kow to generate reactive oxygen species (ROS) and cause oxidative stress as a side effect. This result in higher lipid peroxidation, explaining its high value in this assay. On the other hand, Silymarin and hesperidin are natural antioxidants. They scavenge free radicals and protect membranes from oxidative damage,

resulting in lower lipid peroxidation. As the values of p for silymarin, hesperidin and doxorubicin treatment in LPO assay on MDA-MB 231 cell line are less than 0.01 level of sigificance, so, results are statistically significant. As the results are statistically significant, so it can be said that observed results are not due to chance, but establishes prooxidant effect of doxorubicin while protective role of silymarin and hesperidin in reducing oxidative stress.

6.5.6.3- Catalase (CAT) activity:

Catalase is an enzyme which converts harmful hydrogen peroxide gasinto water and oxygen gas. This is a colorimetric assay performed to assess catalase activity when cells MDA-MB 231 were treated with natural compounds silymarin, hesperidin and standard drug doxorubicin. The result of catalase activity obtained, when cells were treated with these compounds at concentrations 6µM and 12µM respectively, was as 26.476±1.123, 16.112±0.462, 12.741±0.816, 51.484±0.760, 59.139±1.651, 112.914±2.848, 179.557±16.755.

Table 6.18: Showing Catalase activity in MDA-MB 231 cells when treated with control, doxorubicin, silymarin and hesperidin at concentrations 6µM and 12µM.

Concentration	Catalase activity
Control	26.476±1.123
Doxorubicin 6 µM	16.112±0.462
Doxorubicin 12 µM	12.741±0.816
Silymarin 6 µM	51.484±0.760
Silymarin 12 µM	59.139±1.651
Hesperidin 6 µM	112.914±2.848
Hesperidin 12 µM	179.557±16.755

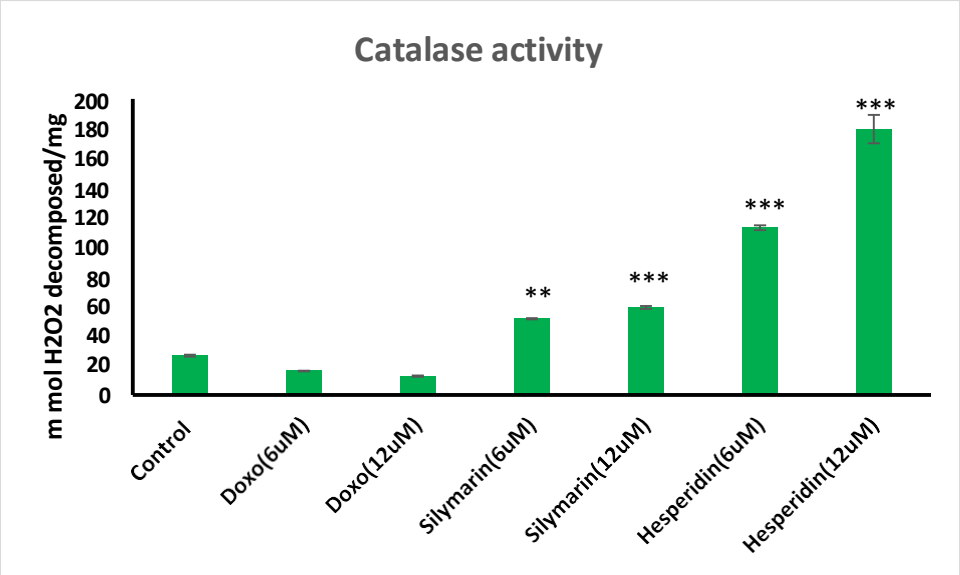


Figure 6.19: Showing result of catalase activity when MDA-MB 231 cells were treated with different natural compounds. Values are means ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001 when in comparison to control group.**

Doxorubicin generates excessive free radicals but it reduces catalase activity due to doxorubicin induced oxidative stress which overwhelms antioxidant system, inhibiting catalase enzyme. Suppression of catalase shows failure of antioxidant defense under high oxidative stress caused by doxorubicin. Silymarin and hesperidin being natural compounds does not cause oxidative stress and increase catalase activity. Hesperidin has highest catalase activity, shows it has highest antioxidant properties among all compounds, with highest level of significance. As the values of p for silymarin, hesperidin treatment in catalase assay on MDA-MB 231 cell line are less than 0.01 level of sigificance, so, results are statistically significant. These statistically significant differences indicate that the observed increases in catalase activity with hesperidin and silymarin are not due to random chance and reflect real biological effects. Hesperidin's significantly higher catalase activity suggests it strongly enhances antioxidant defense mechanism in MDA-MB 231 cells, while doxorubin's significantly lower catalase activity confirms its known role in inducing oxidative stress and depleting cellular defense enzymes.

6.5.6.4- Superoxide dismutase (SOD) activity-

SOD is an antioxidant enzyme which decreases oxidative stress produced by metabolic reactions in our cells (Beyer et al., 1991). This is a colorimetric assay performed to assess SOD activity when cells MDA-MB 231 were treated with natural compounds silymarin, hesperidin

and standard drug doxorubicin. The result of SOD activity obtained, when cells were treated with these compounds at concentrations 6µM and 12µM respectively, was as 133.328±16.666, 132.711±6.172, 171.332±62.302, 60.057±15.014,56.687±6.32, 273.926±22.789, 353.521±75.754.

Table 6.19: Showing SOD activity in MDA-MB 231 cells when treated with control, doxorubicin, silymarin and hesperidin at concentrations 6µM and 12µM.

Concentration	SOD activity
Control	133.328±16.666
Doxorubicin 6 µM	132.711±6.172
Doxorubicin 12 µM	171.332±62.302
Silymarin 6 µM	60.057±15.014
Silymarin 12 µM	56.687±6.32
Hesperidin 6 µM	273.926±22.789
Hesperidin 12 µM	353.521±75.754

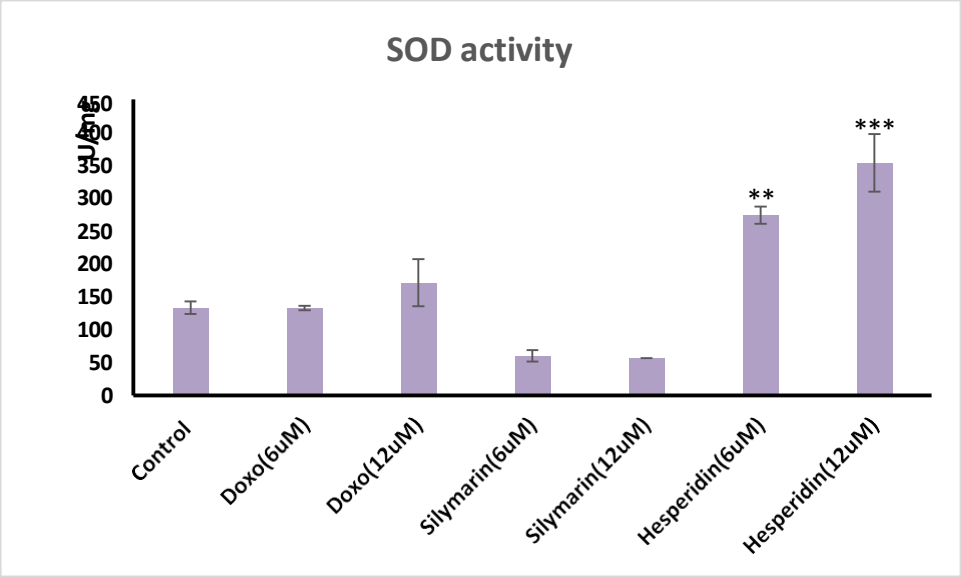


Figure 6.20: Showing result of SOD activity when MDA-MB 231 cells were treated with different natural compounds. Values are means ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001 when in comparison to control group**

In this result, hesperidin has highest SOD activity as it might be stimulating endogenous antioxidant defense mechanism, thus upregulatig SOD activity. Its antioxidant action could be partly indirect, promoting body's own antioxidant system, while doxorubicin being chemotherapy drug generates oxidative stress, so cells may compensate this stress by increasing SOD activity to some extent. So, observed increase in SOD activity may be cellular defense from free radical, not a direct benefit of doxorubicin. However, silymarin showing least SOD activity as it scavenges free radicals mainly by GSH and catalase, not always SOD. Low SOD can be due to dose dependent effect as low or high doses may not stimulate SOD optimally. Bioavalability, poor soluility or formulation issue can also be there. Silymarin showed less activity than doxorubicin due to differences in their chemical behavior and mechanism of antioxidant action. Quinone structure of doxorubicin participate in redox cycling, which can artificially enhance SOD like activity in some assays. However, silymarin works by enhancing endogenous antioxidant defense over time or chelating metal ions actionns that may not strongly affect the superoxide radical specifically in short term in vitro assay. It also lacks redox active groups like doxorubicin that might directly mimic or enhance SOD activity. Since p value is less than 0.001 for hesperidin, so result is statistically significant, that means higher SOD activity is not due to chance.

6.5.7- Western blotting-

Western blotting is a technique used for protein analysis, basically to identify and check protein expression. For PPAR-γ to work against triple negative breast cancer, its expression must increase to express its anticancer effects. So, changes in PPAR-γ expression was assessed at two different concentrations of natural PPAR-γ agonist silymarin and hesperidin in comparision to synthetic PPAR-γ agonist pioglitazone and standard drug doxorubicin. Expression of PPAR-γ was compared with standard house keeping protein Actin.An increase in protein expression of PPAR-γ was observed after treatment with pioglitazone at concentration 24µM, while silymarin being a partial agonist of PPAR-γ slightly increase protein expression of PPAR-γ at both concentrations 12µMand 24µM. For doxorubicin on the other hand, no such increase in the protein expression of PPAR-γ was observed at concentration 24µM (Table 26, Figure 46).

Table 6.20: Showing result of western blotting, assessing PPAR-γ expression in MDA-MB 231 cells while treatment with Silymarin and other test compounds

Concentration	PPAR-γ expression w.r.t. % of Control
Control	100±0
Pioglitazone (24 µM)	221.5±11
Silymarin (12 µM)	121±3
Silymarin (24 µM)	136±4
Doxorubicin(24 µM)	87.5±4.5

The data is provided as mean±SD of 3 independent tests.

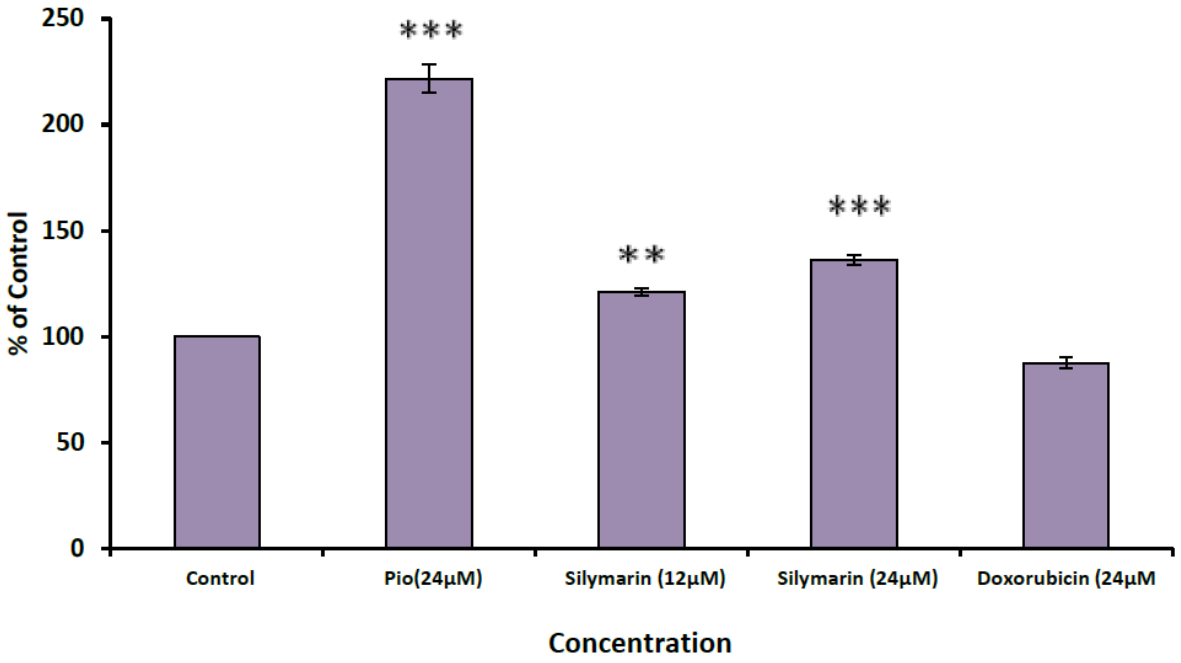


Figure 6.21: Showing result of western blotting in which PPAR-γ expression was assessed when MDA-MB 231 cells were treated with two concentrations of silymarin. Values are means ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001 when in comparison to control group.**

As the value of p for silymarin and pioglitazone treatment in western blotting on MDA-MB 231 cell line are less than 0.01 level of sigificance, so, results are statistically significant.

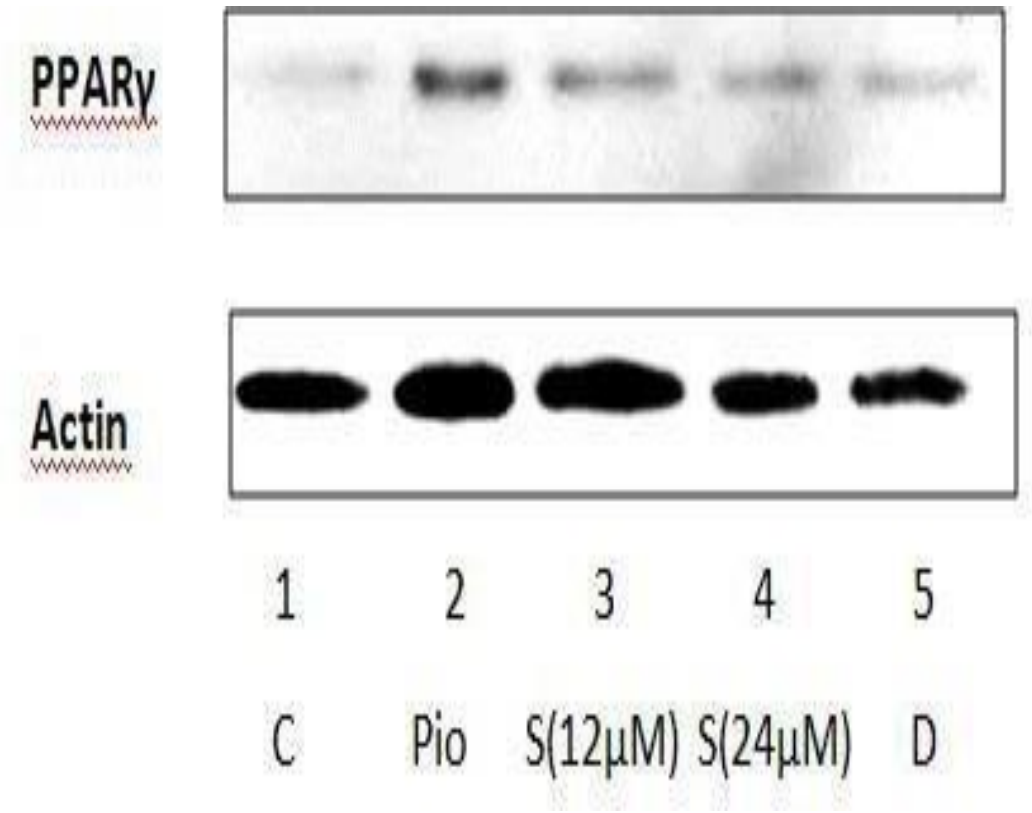


Figure 6.22: Western blot of PPAR-γ expression in MDA-MB 231 cell line after treatment with silymarin at two different concentrations. Lane 1: Control (24 µM). Lane2 Pioglitazone (24 µM). Lane 3: Silymarin (12 µM). Lane 4: Silymarin (24 µM) Lane 5: Doxorubicin (24 µM) Values are mean±SD from three separate blots.

Whereas, the result of western blotting performed with hesperidin as test compound was also similar. An increase in protein expression of PPAR-γ was observed after treatment with pioglitazone at concentration 24µM, while hesperidin being a partial agonist of PPAR-γ slightly increase protein expression of PPAR-γ at both concentrations 12µM and 24µM. For doxorubicin on the other hand, no such increase in the protein expression of PPAR-γ was observed at concentration 24µM (Table 27, Figure 48).

Table 6.21: Showing result of western blotting, assessing PPAR-γ expression in MDA-MB 231 cells while treatment with Hesperidin and other test compounds

Concentration	PPAR-γ expressionw.r.t. % of control
Control	100±0
Pioglitazone (24 µM)	236±42
Hesperidin (12 µM)	144±12
Hesperidin (24 µM)	190.5±10.5
Doxorubicin(24 µM)	75.5±6.5

The data is provided as mean±SD of 3 independent tests.

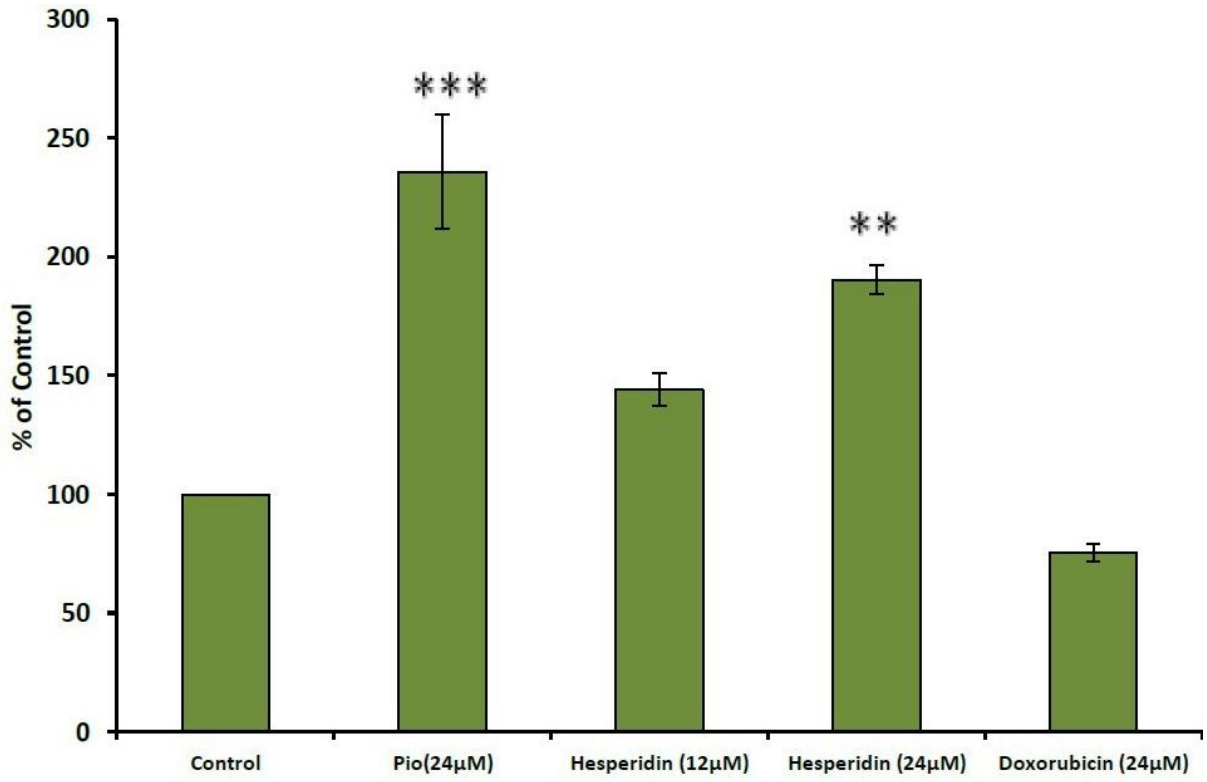


Figure 6.23: Showing result of western blotting in which PPAR-γ expression was assessed when MDA-MB 231 cells were treated with two concentrations of Hesperidin. Values are means ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001 when in comparison to control group.**

As the values of p for hesperidin and pioglitazone treatment in western blotting on MDA-MB 231 cell line are less than 0.01 level of sigificance, so, results are statistically significant.

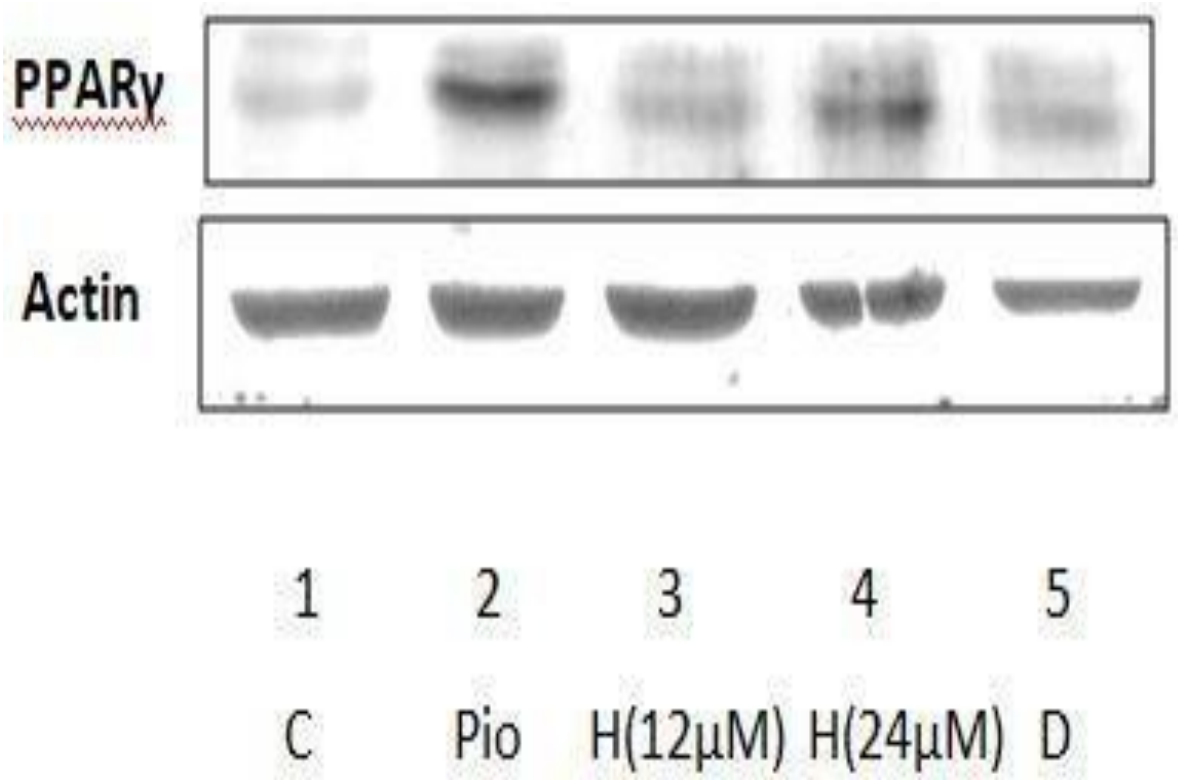


Figure 6.24: Western blot of PPAR-γ expression in MDA-MB 231 cell line after treatment with Hesperidin at two different concentrations. Lane 1: Control (24 µM). Lane 2 Pioglitazone (24 µM). Lane 3: Silymarin (12 µM). Lane 4: Silymarin (24 µM) Lane 5: Doxorubicin (24 µM) Values are mean±SD from three separate blots.

Pioglitazone belongs to the thiazolidinedione (TZD) drugs acts as full agonist and binds directly and completely to PPAR-γ and activates it fully. However, Silymarin being natural compound activates indirectly by reducing oxidative stress and inflammation, which in turn increase PPAR- γ expression. It does not bind completely as it is partial agonist thus its effect is weaker and slower, leading to moderate expression on western blot. On the other hand, doxorubicin, being a cytotoxic chemotherapy drug, does not increase PPAR-γ expression. In fact, it may reduce PPAR-γ expression due to induced oxidative stress. Thatswhy least PPAR-γ expression show by it. Results are statistically significant as p value is less than .01 for silymarin ad hesperidin and less than .001 for pioglitazone. This shows that these results are not due to chance.

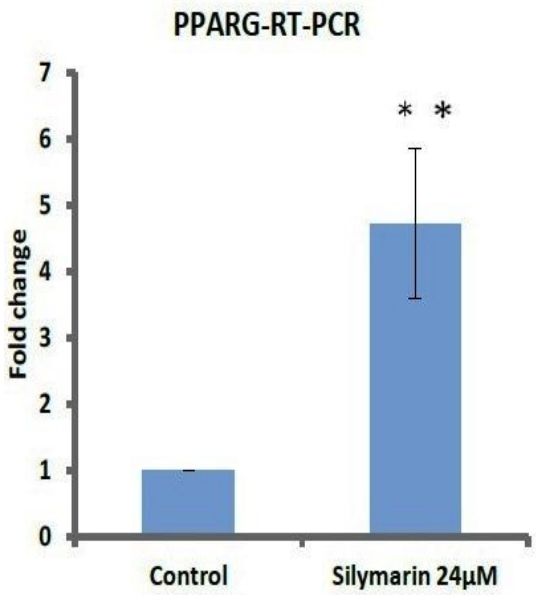
6.5.8- Real time PCR

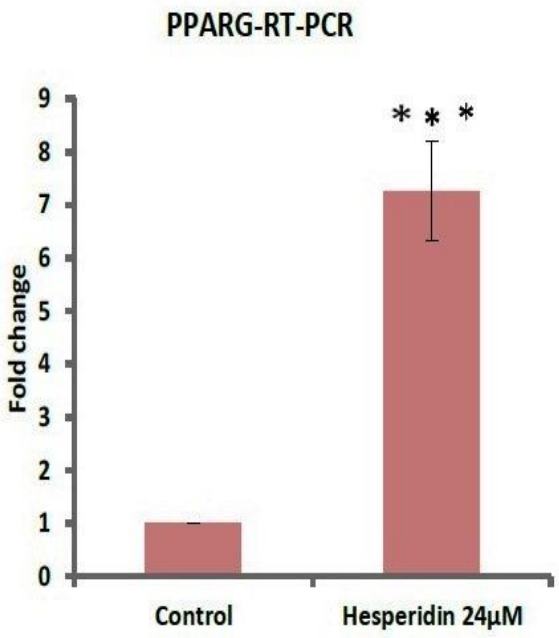
Real time PCR was performed on mRNA extracted from MDA-MB 231 cells for evaluating the expression of different genes in consideration like p53, BCL2, BAX, etc., after treatment with silymarin and hesperidin. The result obtained is tabulated as follows:

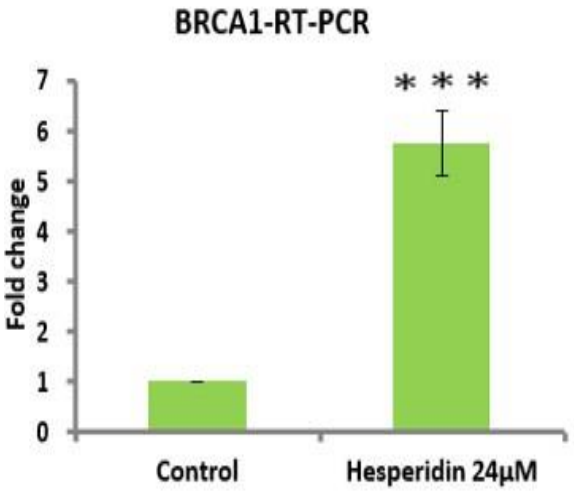
Table 6.22: Showing expression of different genes after treatment with silymarin and hesperidin

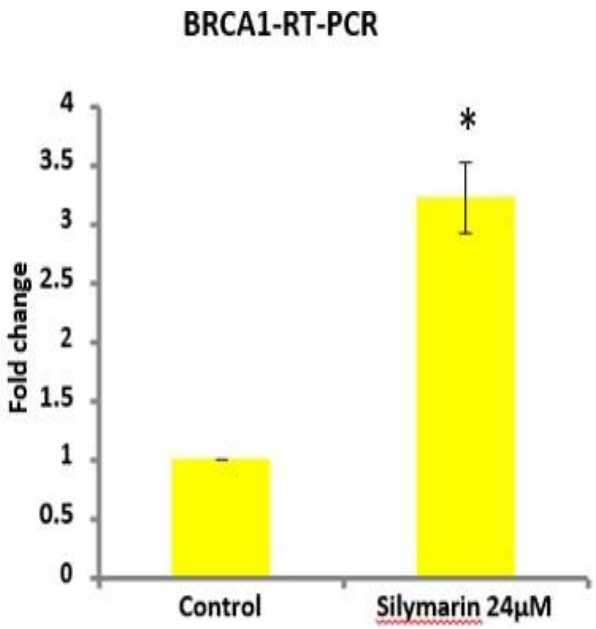
Gene	Silymarin treatment	Hesperidin treatement
PPARG	4.72±1.13	7.25±0.94
BRCA1	3.22±0.30	5.75±0.65
P53	3.42±0.56	6.91±0.78
SDC1	1.97±0.19	2.56±0.40
BCL2	-2.63±0.18	-6.25±1.36
BAX	-2.21±0.09	-2.65±0.52

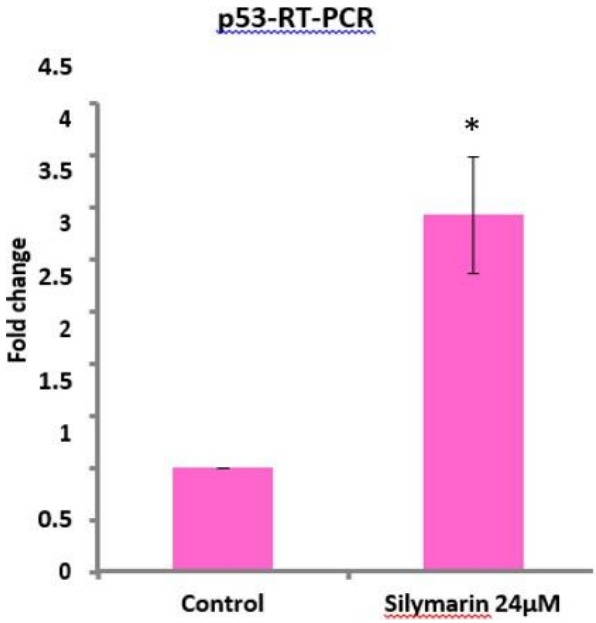
The data is provided as mean±SEM of 3 independent tests.

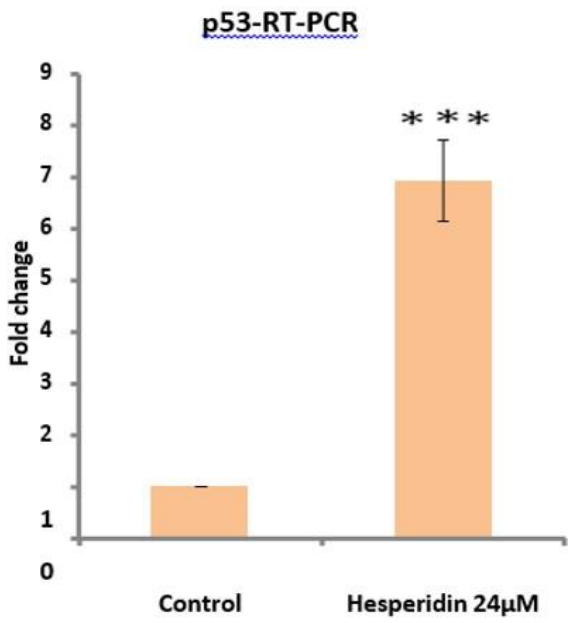

PPARG-RT-PCR
Fold change
0
1
2
3
4
5
6
7
* *
Control
Silymarin 24µM

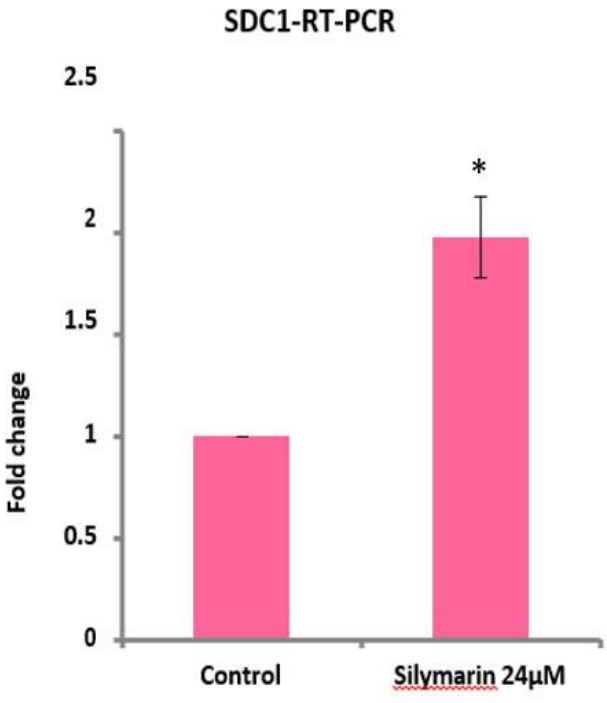

PPARG-RT-PCR
Fold change
0
1
2
3
4
5
6
7
8
9
* * *
Control
Hesperidin 24µM

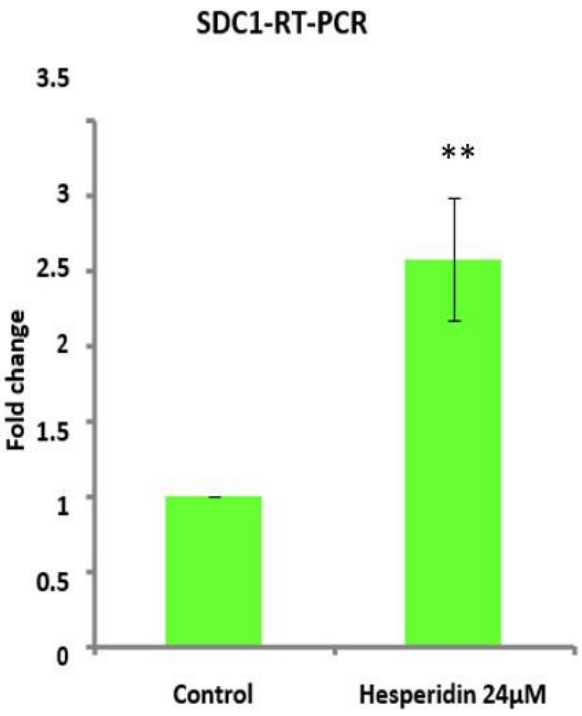

BRCA1-RT-PCR
Fold change
0
1
2
3
4
5
6
7
* * *
Control
Hesperidin 24µM


BRCA1-RT-PCR
Fold change
0
0.5
1
1.5
2
2.5
3
3.5
4
*
Control
Silymarin 24µM


p53-RT-PCR
Fold change
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
Silymarin 24µM


p53-RT-PCR
Fold change
9
8
7
6
5
4
3
2
1
0
* * *
Control
Hesperidin 24µM


SDC1-RT-PCR
Fold change
2.5
2
1.5
1
0.5
0
*
Control
Silymarin 24µM


SDC1-RT-PCR
Fold change
3.5
3
2.5
2
1.5
1
0.5
0
**
Control
Hesperidin 24µM

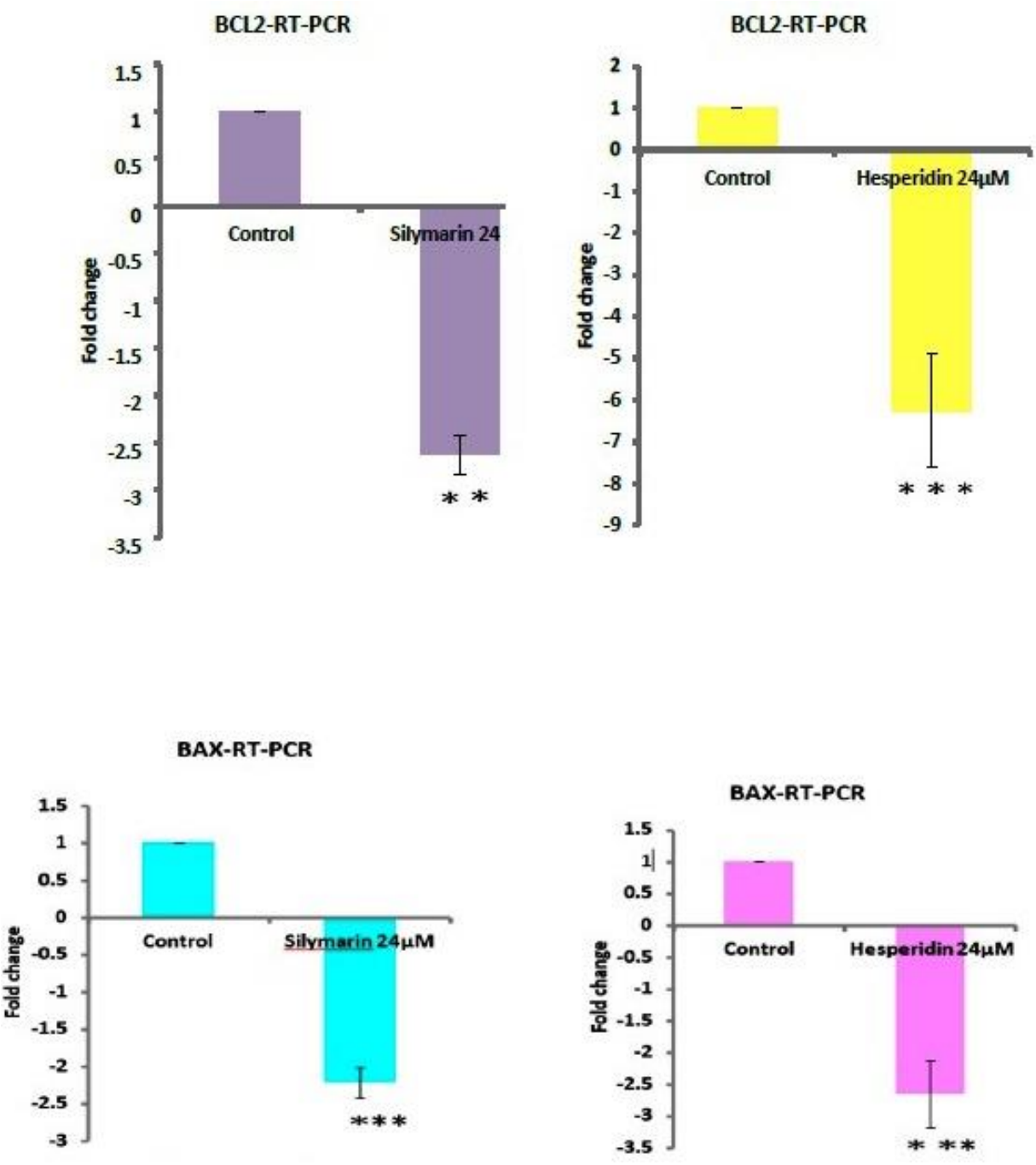


Figure 6.25: Showing upregulation of genes involved in decreasing proliferation of cancer cells and increasing apoptosis upon treatment of cancer cells (MDA-MB 231 cell line) with silymarin and hesperidin. Valuesare means ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001 when in comparison to control group**

Both compounds silymarin and hesperidin enhance lipid metabolism, anti inflammatory response and tumor suppression. Upregulation of PPARG supports cell differentiation and inhibit tumor progression. Increased expression of BRCA1 shows increased genomic stability and protective anticancer effect. Increased expression of p53 shows both compounds may activate tumor suppressive pathways. Increased expression of SDC1 inndicate improved epithelial barrier integrity. Whereas downnregulation of BCL2 and BAX gene shows proapoptotic potential of both silymarin and hesperidin. This may be as intrinsic apoptosis is not dominant pathway being activated or that regulation is occurring post-trannscriptionally or is cell-type/time dependent. Genes like PPARG, BRCA1, p53 and SDC1 show significant upregulation, with p values less than 0.05, confirming that the treatments likely caused real, biological relevant changes, not by chance. BCL2 and BAX downregulation is also statistically significant, supporting its potential role in proapoptotic signaling. As the values of p for silymarin and hesperidin treatment in Real time PCR on MDA-MB 231 cell line are less than 0.05 level of sigificance, so, results are statistically significant.

6.6- Invivo studies-

Invivo studies were done on fertilized chicken eggs to assess potential of test compounds silymarin and hesperidin to reduce angiogenesis by performing CAM angiogenesis assay, and to assess the potential of hesperidin to reduce metastasis by performing Cell invasion assay.

6.6.1- CAM angiogenesis assay-

As the result of CAM angiogenesis assay is showing silymarin at 3 µg concentration decreased angiogenesis and length of blood vessels was found upto 450 mm but, at 6 µg it decreased more angiogenesis and length of blood vessels was then found to be upto 300 mm. Hesperidin however at 3 µg itseself decreased angiogenesis in better extent and the length of blood vessels was found to be about 50 mm and at 6 µg concentration hesperidin completely inhibited blood vessel formation like positive control doxorubicin. This is shown in fig 6.32.

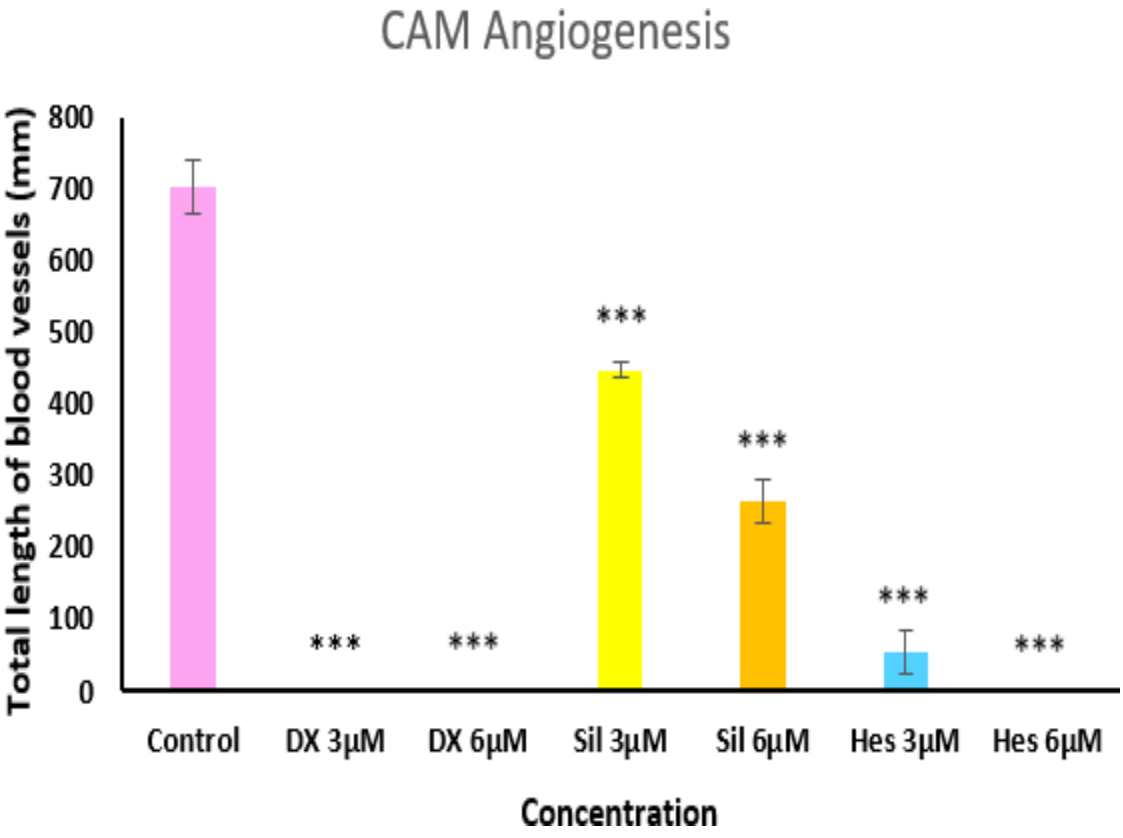


Figure 6.26: Showing CAM angiogenesis assay result upon treatment with different concentration of doxorubicin, silymarin and hesperidin w.r.t. control. Values are mean ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001 whenin comparison to control group**

Negative control showed 0% inhibition however positive control doxorubicin showed 100% inhibition. Silymarin at concentration 3 µg showed less than 40% inhibition while at 6 µg inhibitionwas 60%. However, with hesperidin at 3 µg concentration inhibition was 90% and at 6 µg concentration inhibition was 100% like positive control doxorubicin. This is shown in fig 6.33. Hesperidin demonstrates strong inhibitory potential, possibly rivaling doxorubicin, while silymarin shows moderate, dose-dependent activity. These results suggest hesperidin may be a more promising candidate for further development, especially considering its high efficacy at low concentrations. Decrease in the length of blood vessels formed on treatment with silymarin, hesperidin and doxorubicin is statistically significant w.r.t. control as p value is less than 0.001. Moreover, Hesperidin is significantly more potent than silymarin at the same concentration as the p value is less than 0.001. Silymarin shows dose-dependent, but less potent effects. These statistically significant differences support the conclusion that hesperidin is a

more effective inhibitor of angiogenesis than silymarin under the tested conditions. As the values of p for silymarin, hesperidin and doxorubicin treatment in CAM angiogenesis assay on MDA-MB 231 cell line are less than 0.001 level of sigificance, so, results are statistically significant.

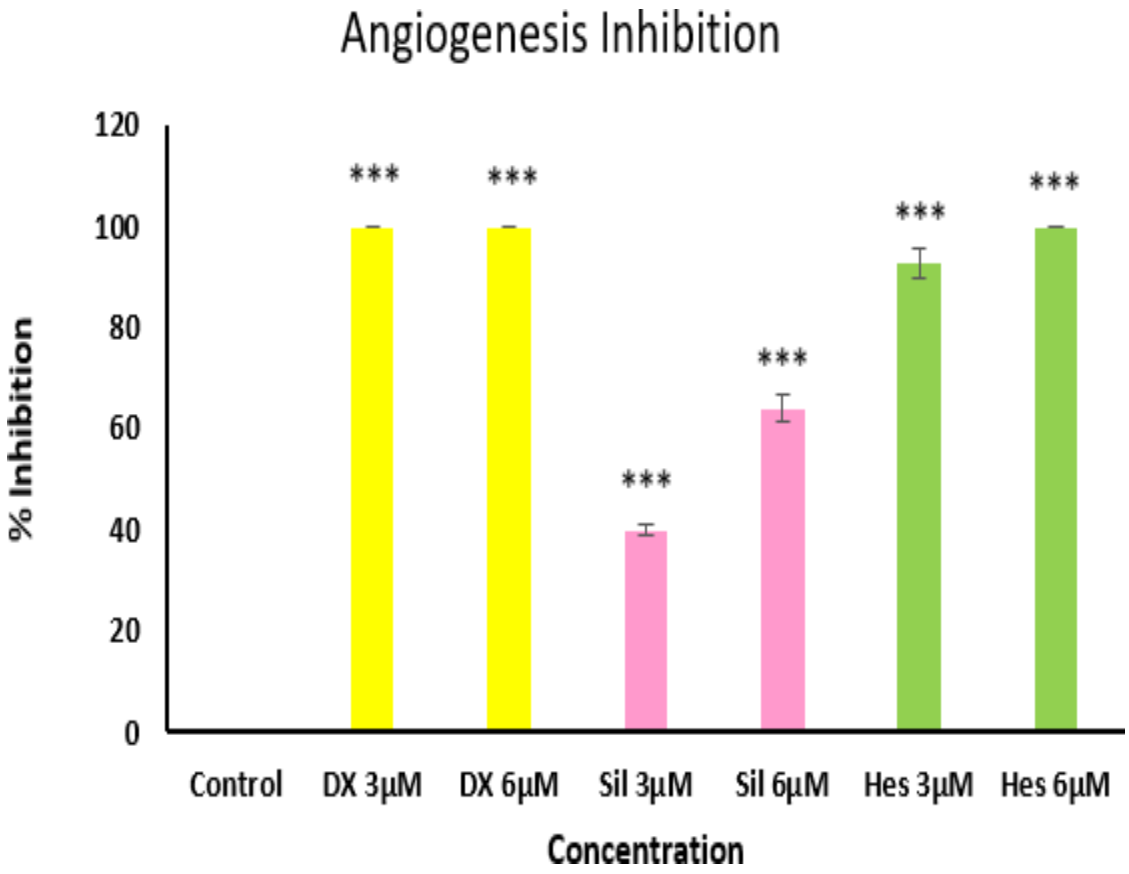


Figure 6.27: Showing angiogenesis inhibition upon treatment with different concentration of doxorubicin, silymarin and hesperidin w.r.t. control. Values are mean ±SEM. * symbolizes p<0.05, ** symbolizes p<0.01, * symbolizes p<0.001 whenin comparison to control group**

In the CAM angiogenesis assay, hesperidin demonstrated nearly 100% inhibition of blood vessel formation, showing a comparable effect to doxorubicin, a well-established chemotherapeutic agent. This strong inhibitory effect by hesperidin was statistically significant, with a p-value < 0.001, indicating a high level of confidence that the observed effect was not due to random chance. In contrast, silymarin exhibited a noticeably lower level of angiogenesis inhibition, suggesting less potent anti-angiogenic activity compared to both hesperidin and doxorubicin. The differences between silymarin and the other two compounds were also statistically significant ($p < 0.001$), confirming that the variation in response is meaningful and not due to experimental variability. These results suggest that hesperidin has strong anti-angiogenic potential**,** comparable to that of doxorubicin, and significantly more effective than silymarin. This supports hesperidin's potential as a promising natural compound for anti-cancer therapy targeting angiogenesis. As the values of p for silymarin, hesperidin treatment in CAM angiogenesis assay on MDA-MB 231 cell line are less than 0.001 level of sigificance, so, results are statistically significant.

Below given figure is showing full extent angiogenesis as it is result of negative control.

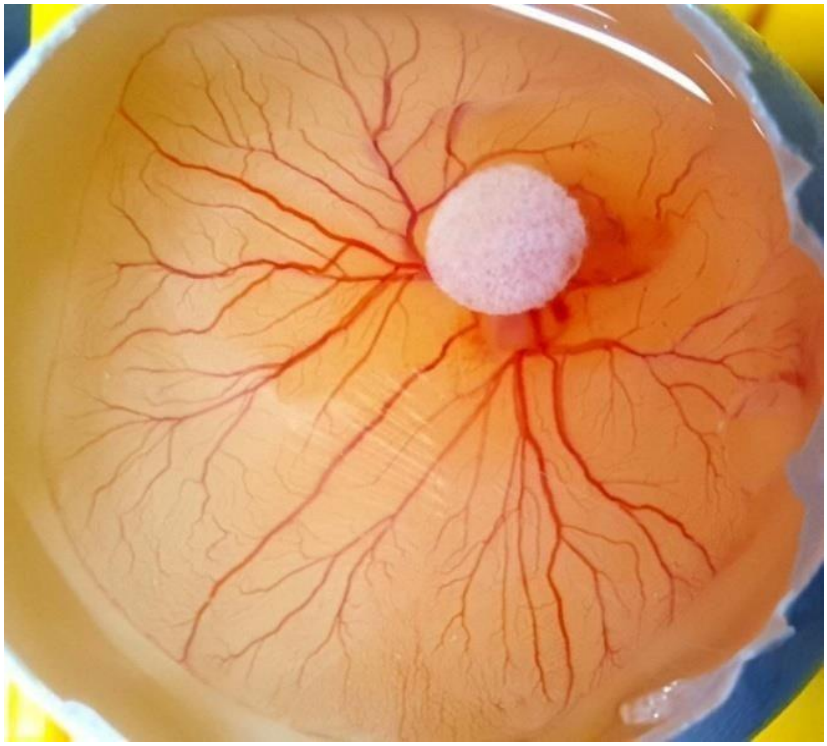


Figure 6.28: Showing angiogenesis at concentration R10 of negative control

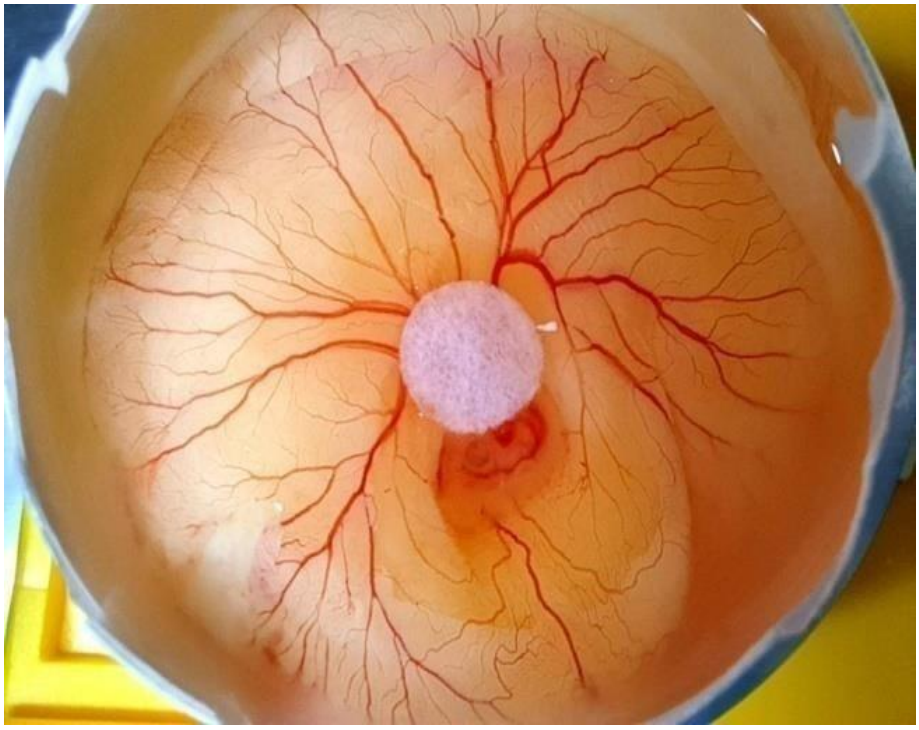


Figure 6.29: Showing angiogenesis at concentration R20 of negative control

However, positive control doxorubicin completely inhibited angiogenesis; result of angiogenesis for doxorubicin is as follows:

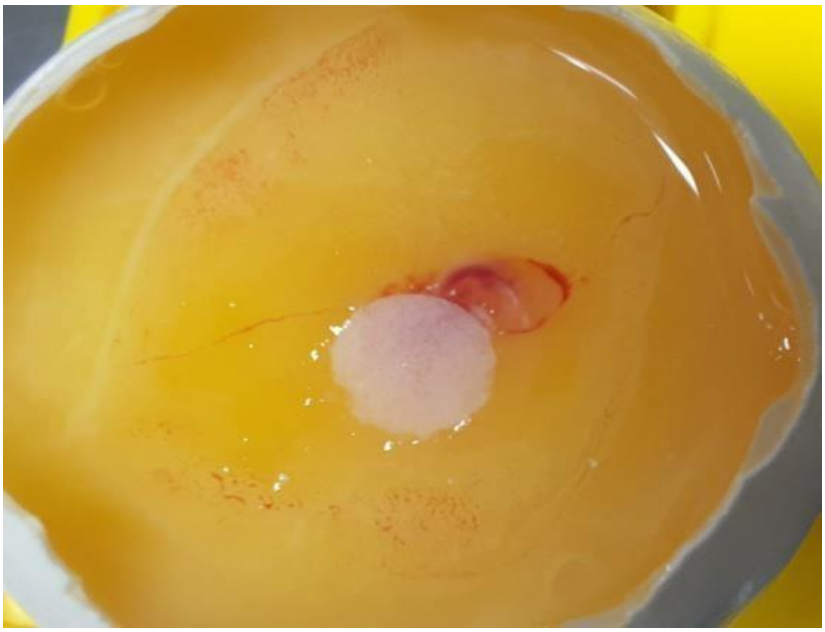


Figure 6.30: Showing angiogenesis at concentration 3 microgram R10 of positive control

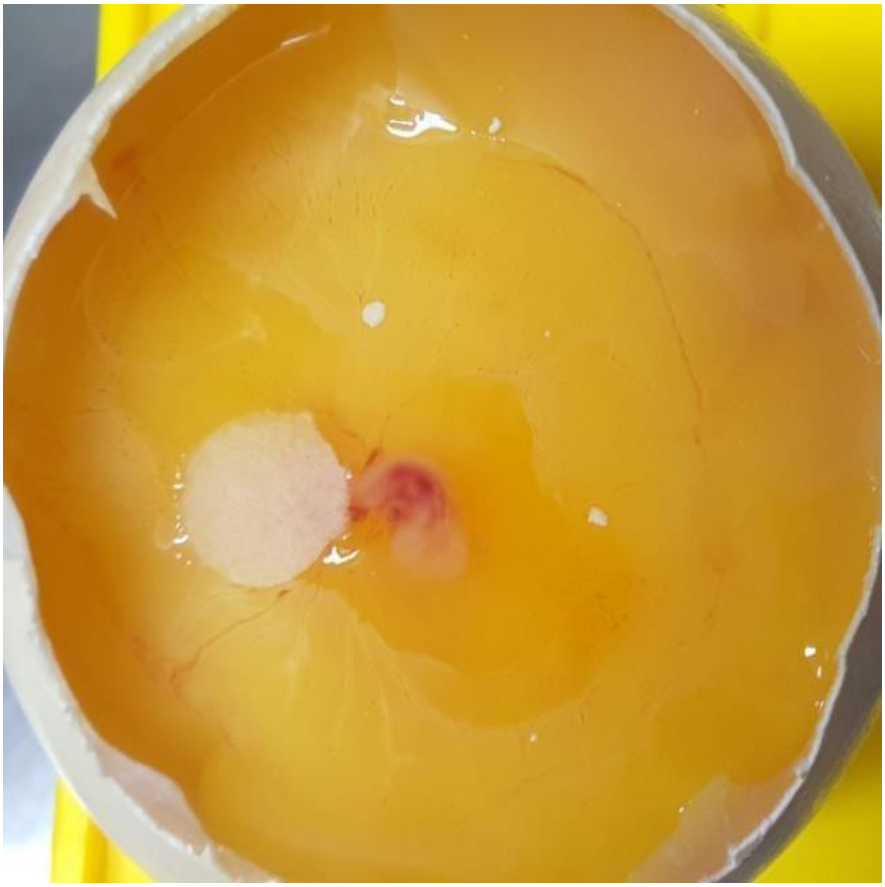


Figure 6.31: Showing angiogenesis at concentration 6 microgram R10 of positive control

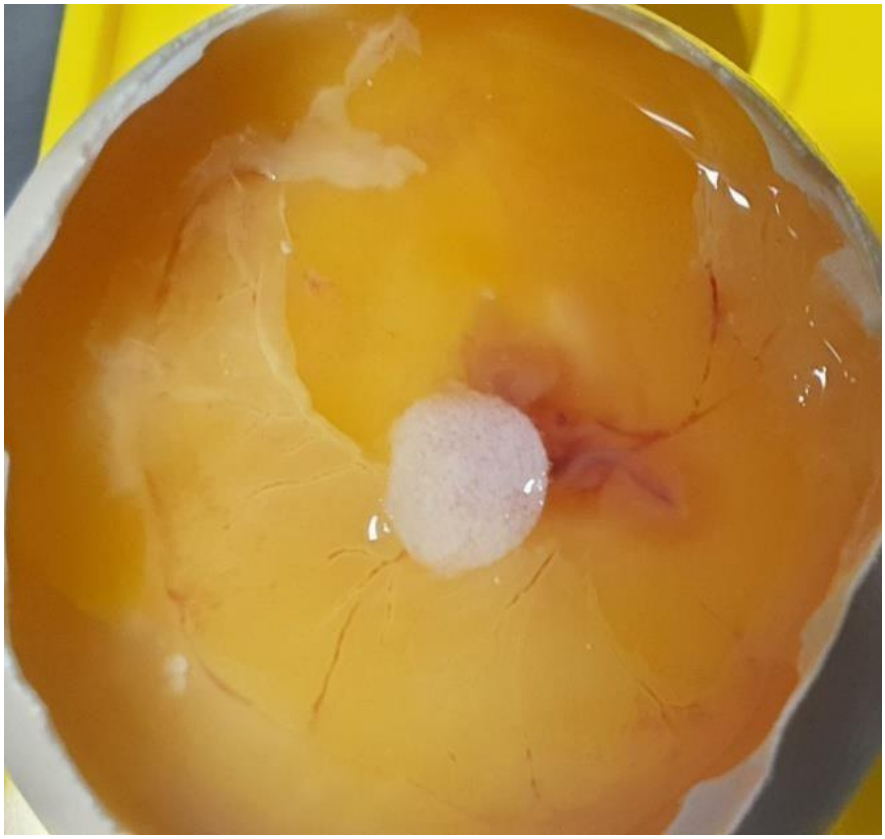


Figure 6.32: Showing angiogenesis at concentration 3 microgram R20 of positive control

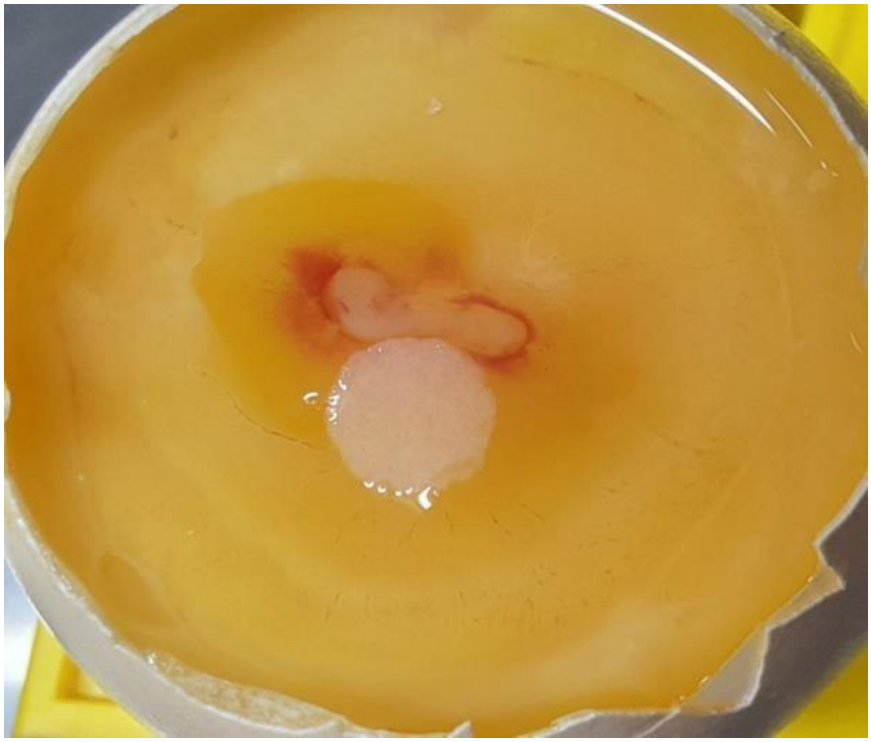


Figure 6.33: Showing angiogenesis at concentration 6 microgram R20 of positive control

However, silymarin inhibited angiogenesis at different concentration in different way. At 3µg concentration it decreased angiogenesis but upto less extent as shown below in fig.

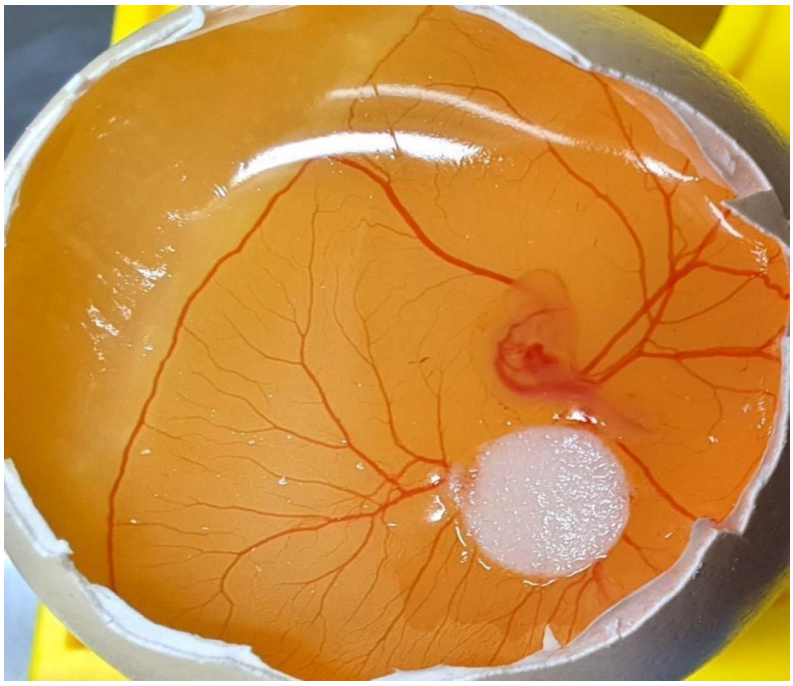


Figure 6.34: Showing angiogenesis at concentration 3 microgram R10 of silymarin.

However, at increased concentration 6µg concentration it decreased angiogenesis more as shown below:

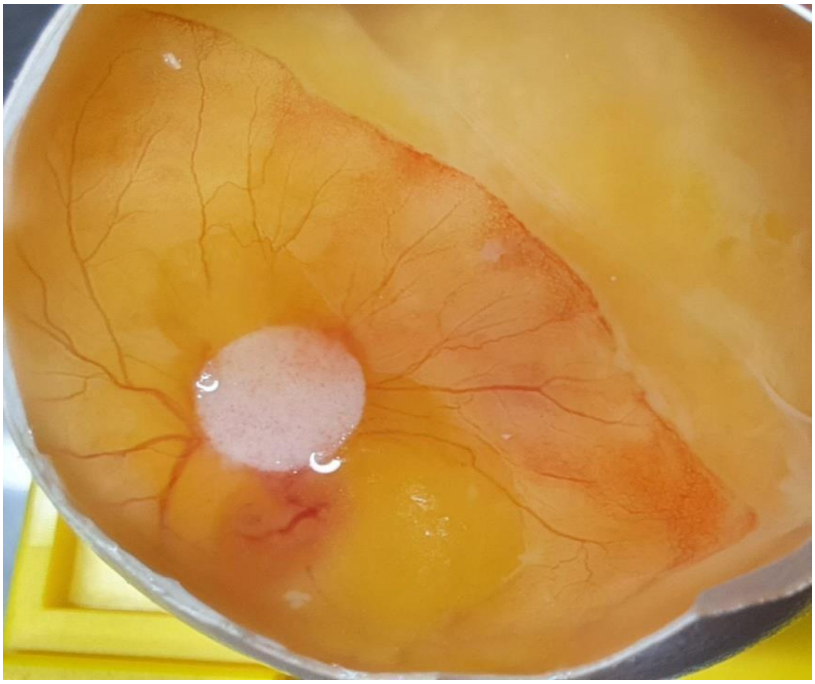


Figure 6.35: Showing angiogenesis at concentration 6 microgram R10 of silymarin.

As experiments were performed in duplicates, so results at both concentration of silymarin are given as follows:

Figure 6.36: Showing angiogenesis at concentration 3 microgram R20 of silymarin.

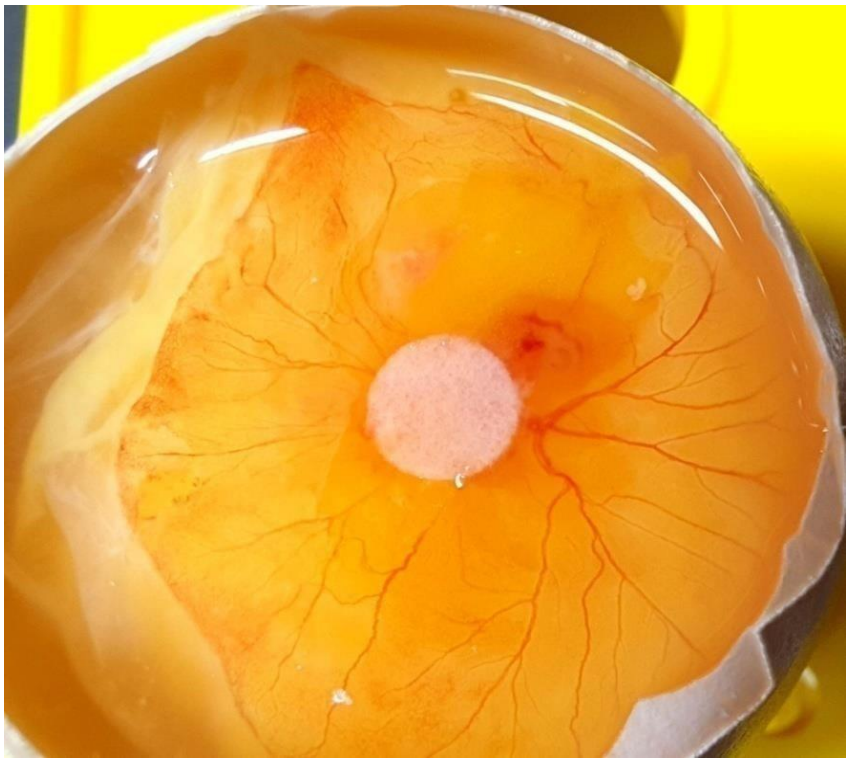


Figure 6.37: Showing angiogenesis at concentration 6 microgram R20 of silymarin.

Similarly we assessed antiangiogenic potential of hesperidin alsoand we found that at 3µg itself hesperidin inhibited blood vessel formation upto 90%, pictorial result is given below.

Figure 6.38 Showing angiogenesis at concentration 3 microgram R10 of Hesperidin.

While at 6µg concentration hesperidin completely inhibited blood vessel formation i.e. showed 100% angiogenesis inhibition like positive control doxorubicin, pictorial result is as follows:

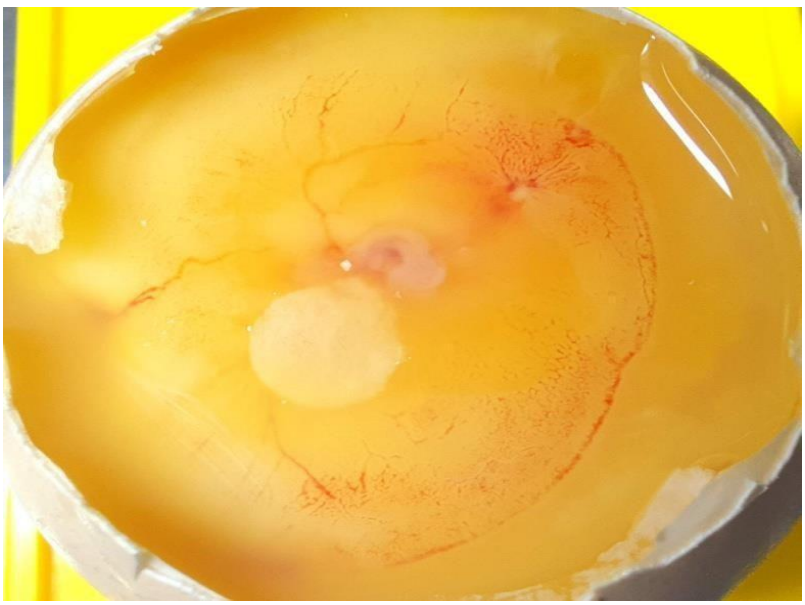


Figure 6.39: Showing angiogenesis at concentration 6 microgram R10 of Hesperidin.

Duplicate results of hesperidin are as follows:

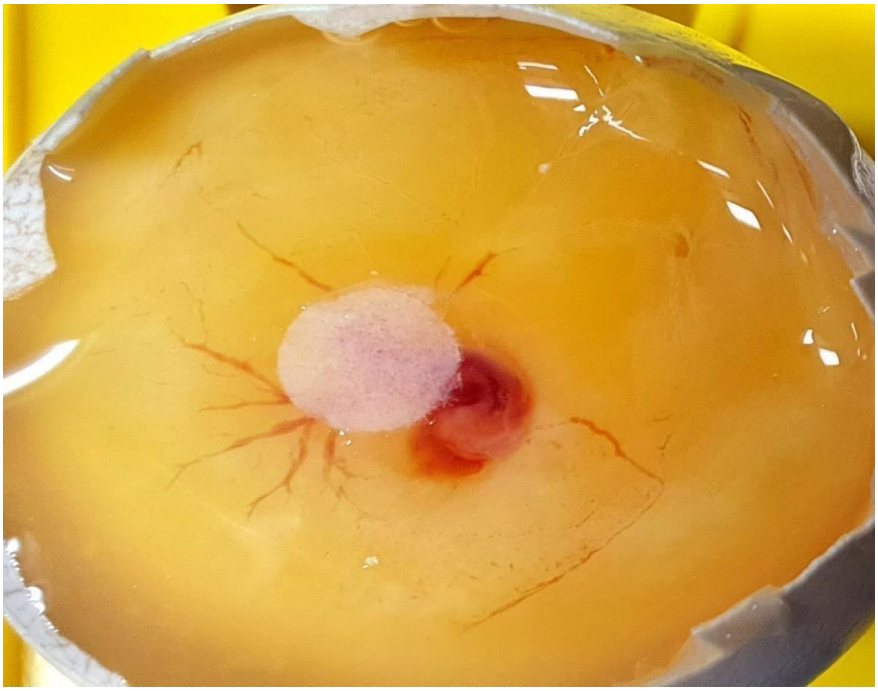


Figure 6.40: Showing angiogenesis at concentration 3 microgram R20 of hesperidin.

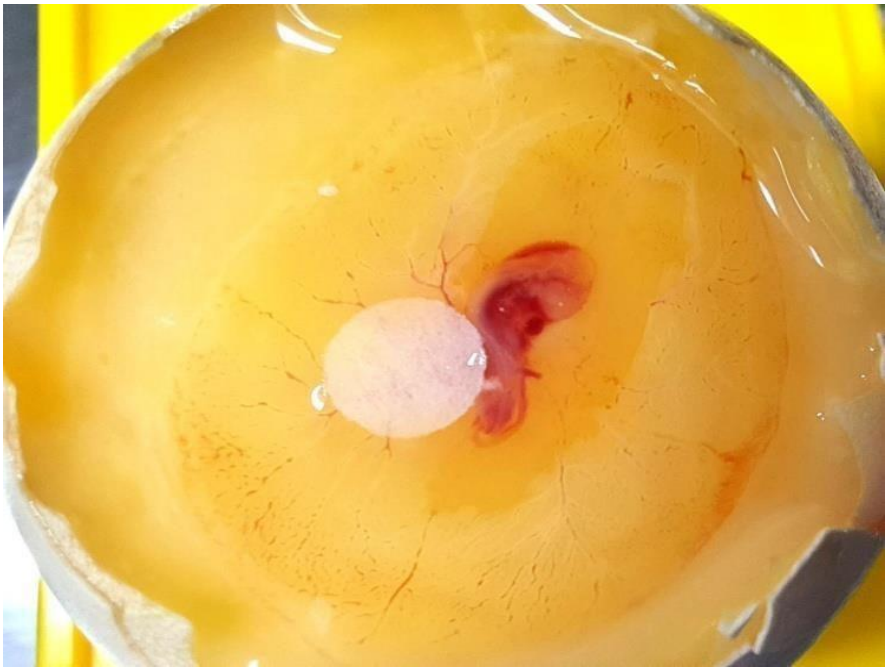


Figure 6.41: Showing angiogenesis at concentration 6 microgram R20 of Hesperidin

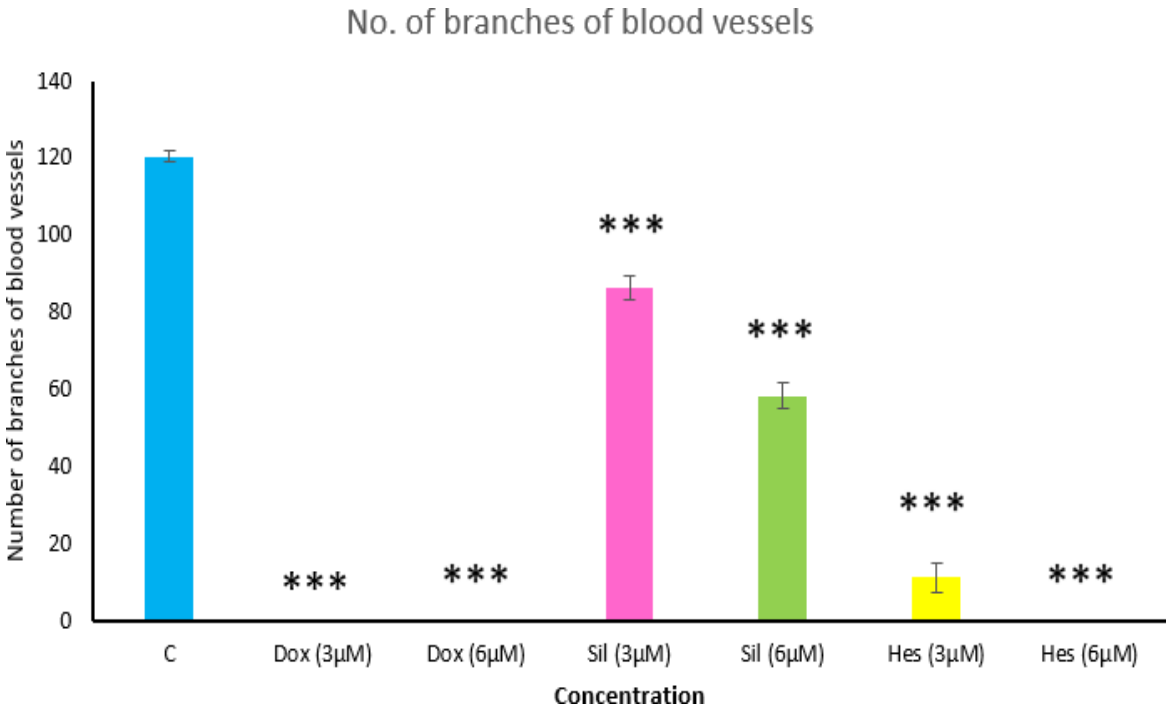


Figure 6.42: Showing decrease in number of branches of blood vessels formed after usage of test compounds in different concentration

All three compounds—doxorubicin, hesperidin, and silymarin—significantly reduced blood vessel formation in the CAM assay. Hesperidin and doxorubicin showed almost identical effects, indicating strong inhibition of angiogenesis. p-value < 0.001 indicates very strong statistical significance (***), suggesting these effects are highly unlikely due to chance. This confirms that the reduction in blood vessel formation by hesperidin and doxorubicin is statistically significant when compared to control (and also when compared to silymarin). Hesperidin's anti-angiogenic effect is statistically equivalent to doxorubicin at higher concentration, with no significant difference between their results. Silymarin, while effective, showed a less pronounced reduction, and the difference between silymarin and hesperidin/doxorubicin is also statistically significant ($p < 0.001$) but less than hesperidin. These results imply that hesperidin could be a potent natural alternative to doxorubicin for inhibiting angiogenesis, with strong statistical backing. Thus, results of CAM angiogenesis assay showed that hesperidin is more potent antiangiogenic and anticancer compound than silymarin. Thus, we chose hesperidin only for further cell invasion assay for proving its antimetastatic nature. As the values of p for silymarin, hesperidin treatment in CAM angiogenesis assay on MDA-MB 231 cell line are less than 0.001 level of sigificance, so, results are statistically significant.

6.6.2- Cell invasion assay-

We performed cell invasion assay also on fertilized chicken eggs and got following results:

Table 6.23: Showing results of cell invasion assay after treatment with Hesperidin

Sample name	Result for Ki67 marker by IHC
Negative Control (Blank)	Strong Positive staining. Indicating large number of intravasated MDA-MB-231 cells
PC (Test concentration – 3µg)	Negative staining. Indicating absence of intravasated MDA-MB-231 cells
Hesperidin –conc1 (Test concentration – 3µg)	Strong Positive staining. Indicating small number of intravasated MDA-MB-231 cells
Hesperidin –conc2 (Test concentration – 6µg)	Negative staining. Indicating absence of intravasated MDA-MB-231 cells

As shown in table, negative control results in large no. of MDA MB-231 cells metastasizing in egg tissues, whilepositive control completely inhibits metastasis of MDA MB- 231 cells andthere is complete absence of intravasation in egg tissues. However, hesperidin at concentration 3µg inhibits metastasis in comparision to negative control but still some cell got metastasized to egg tissues, but hesperidin at concentration 6µg completely inhibited metastasis like positive control did. Pictorial represetation of these findings are as follows:

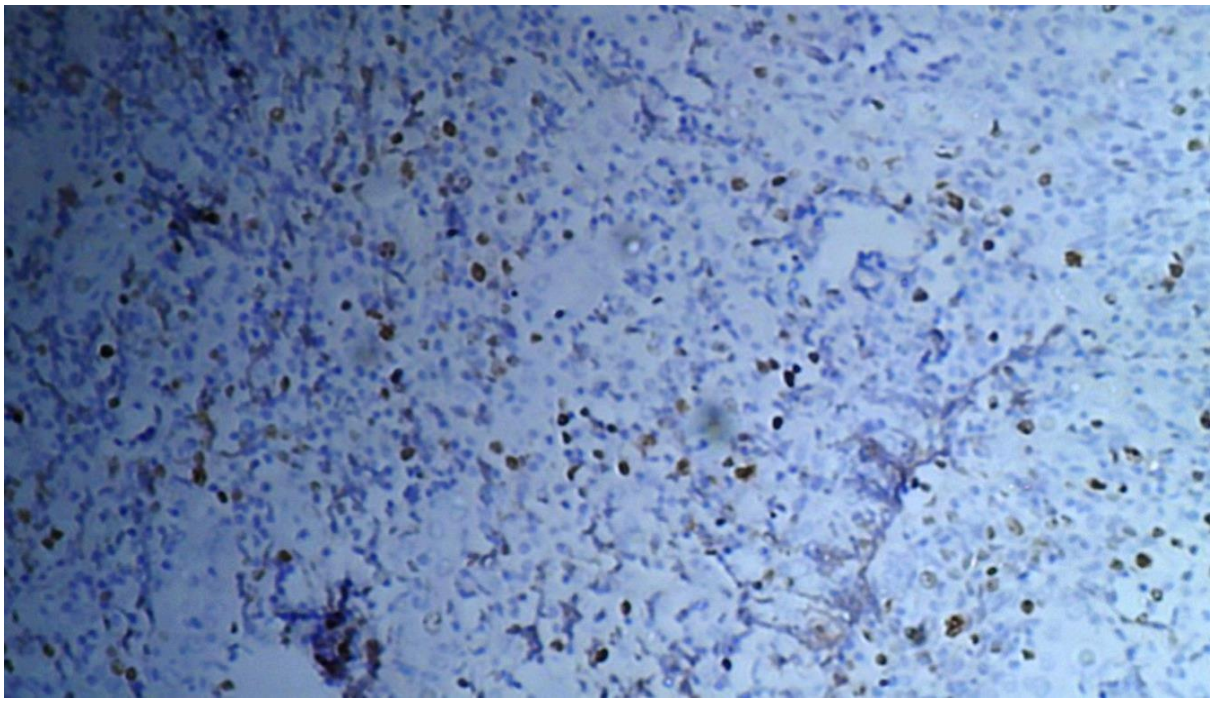


Figure 6.43: Showing metastasis by negative control

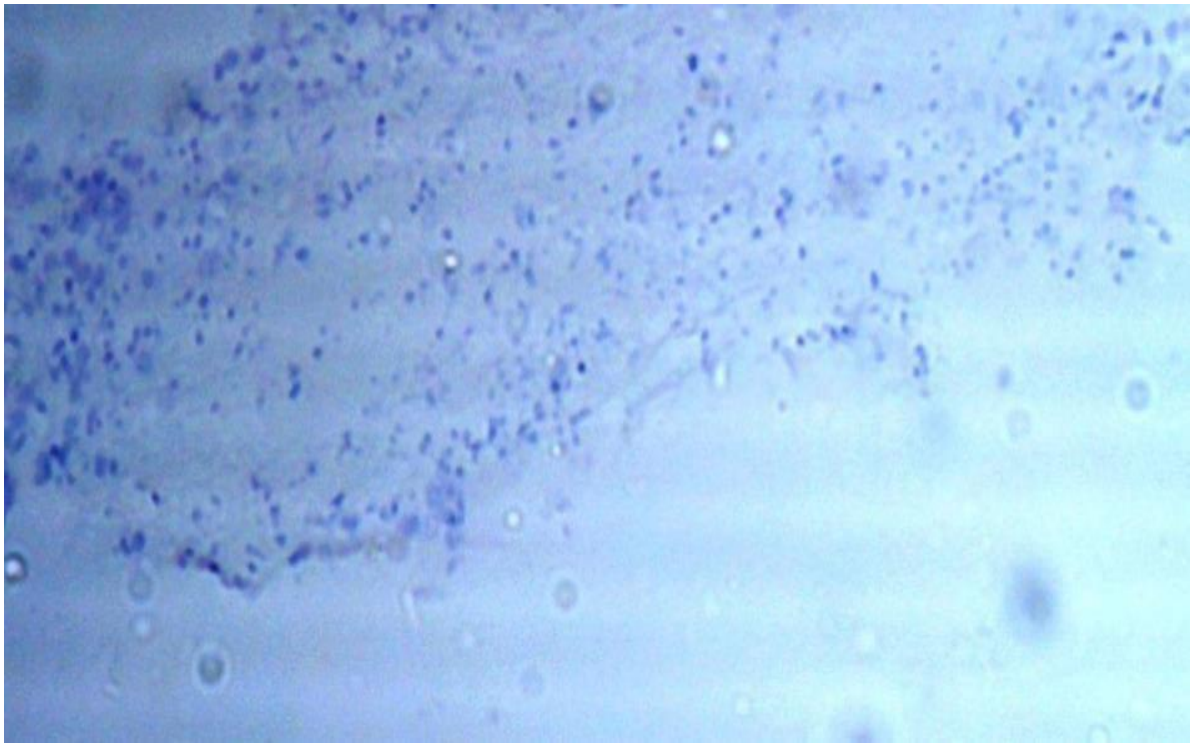


Figure 6.44: Showing complete inhibition of metastasis by positive control

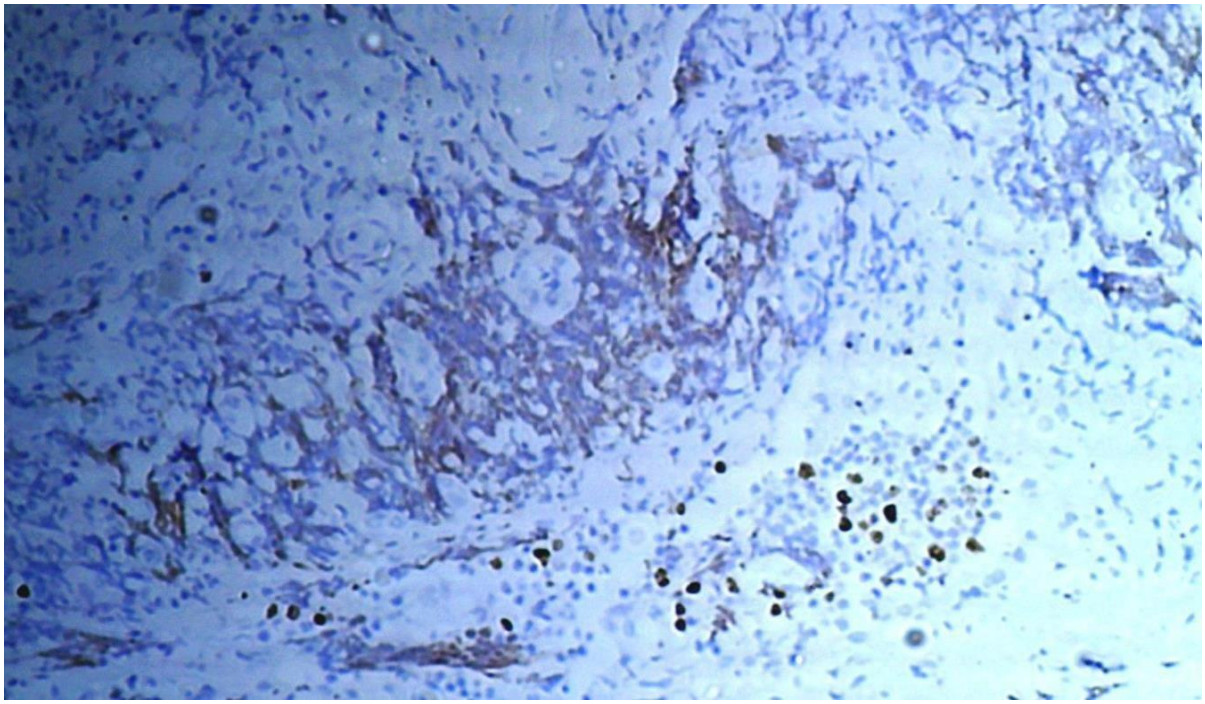


Figure 6.45: Showing less inhibition of metastasis by hesperidin at 3 microgram concentration

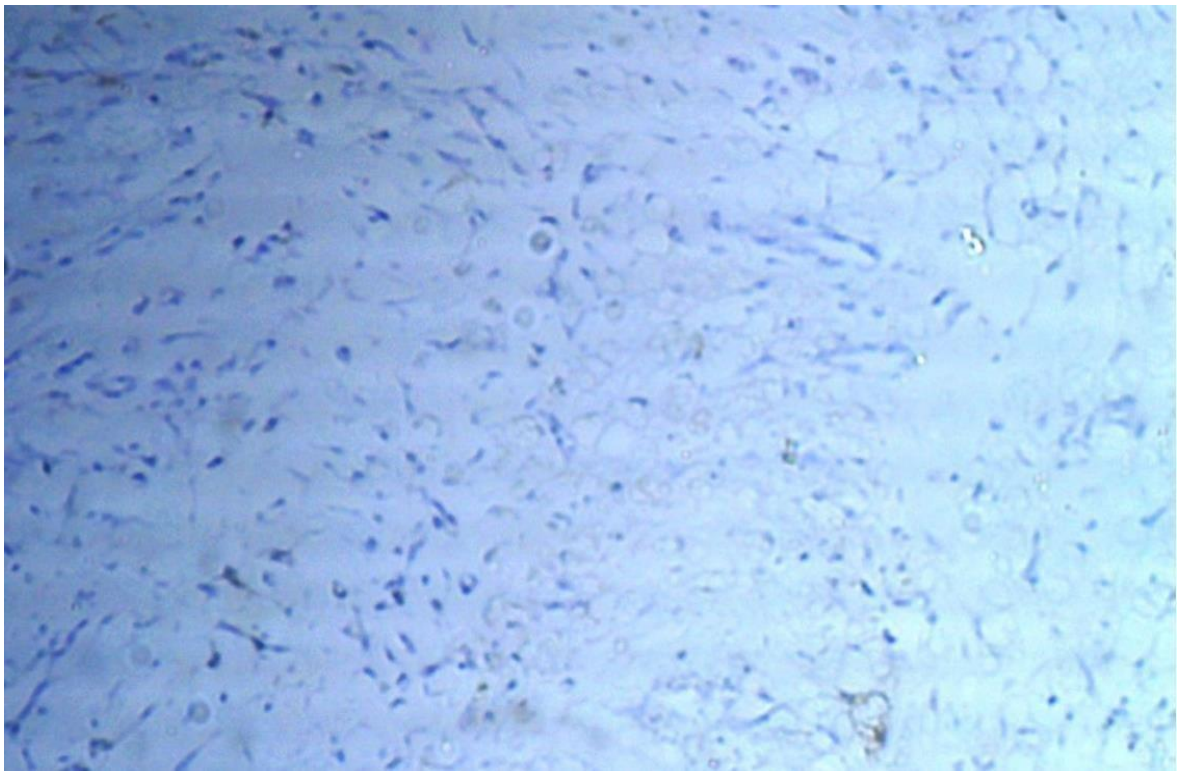


Figure 6.46: Showing complete inhibition of metastasis by hesperidin at 6 microgram concentration

The reduction in invasion at 6 µg of hesperidin is statistically significant compared to both: The negative control (which shows high invasion), and The 3 µg hesperidin group (which shows partial invasion). A p-value < 0.001 implies that the difference in invasion between these groups is highly significant, with less than 0.1% probability that the observed effect is due to chance. The fact that 6 µg hesperidin mirrors doxorubicin (no metastasis) suggests comparable anti-metastatic potential. Hesperidin at 6 µg effectively suppresses metastatic invasion of MDA-MB-231 cells, statistically on par with doxorubicin. The dose-dependent effect of hesperidin is confirmed both biologically and statistically—only partial inhibition at 3 µg, but complete inhibition at 6 µg ($p < 0.001$). This reinforces hesperidin's potential as a natural anti-metastatic agent in TNBC. So, by these results it can be said that hesperidin has very good antimetastatic potential whichis necessary for acting as a therapeutic option against such an aggressive and frequently recurring and highly metastatic breast cancer called TNBC.

CHAPTER 7

DISCUSSION

DISCUSSION

TNBC is a heterogeneous kind of breast cancer, lacking estrogen, progesterone and HER2 neu receptor. It mostly occurs in black and premenopausal women. It is an aggressive tumor, characterized by poor prognosis and metastasis (Zagami P. et al, 2022). Due to lack of major receptors otherwise present in breast cancer, there remains very less options for treatment of TNBC patients like chemotherapy, radiotherapy which comes with various side effects. To avoid side effects, other receptors present in TNBC cells can be considered. PPAR-γ is one such receptor which is expressed in TNBC cells and upon activation by agonist, it poses anticancer effects for various cancers including breast cancer. PPAR-γ kills cancer cells by increasing apoptosis, decreasing proliferation and metastasis. So, for activation of PPAR-γ, synthetic agonists like TZDs have been in usage. However, TZDs being synthetic in nature have very deleterious effects as they fully activate PPAR-γ receptor (Balakumar et al, 2012). Thus, there is need to find natural, potent agonist which can activate PPAR-γ partially, resulting in fewer or no side effects.

Natural phytochemicals, popularly known as neutraceuticals, obtained from various plants are in usage for treating different diseases since Vedic era. Nowadays again the craze for natural phytochemicals is boosted as these compounds cause less or no side effects and side effects are of major concern in the treatment of cancer. So, neutraceuticals are attractive source for drug discovery (Beutler et al, 2009). Therefore, we attempted to explore natural PPAR-γ agonist which can be a potent drug against TNBC.

In our study, we combined three approaches of research namely insilico, invitro and invivo method. In insilico study, we firstly performed similarity searching using already established PPAR-γ agonists as query compounds against diverse and comprehensive database PubChem. Similarity searching is basically based on the notion that if two compounds are structurally similar then their biological properties may also be same (Martin et al., 2002). It is told by Bolton et al., 2011, that if sufficient information about a particular compound is not available then it can be availed from compounds of known similar structure. Thus, it is essential to have proper information about known PPAR-γ compounds having anticancer potential for using their structure in similarity searching to explore and screen newer therapeutic options against TNBC. In this research we used 47 already reported PPAR-γ agonists as query compounds for structure based similarity searching studies in PubChem database. These 47 query compounds were reported in

literature of Wang et al, 2014. These compounds are natural PPAR-γ agonists having anticancer potential, so compounds searched after similarity search were also natural PPAR-γ agonists having anticancer potential of varied extent. In PubChem, the 2D structure based similarity search was performed having more than 80% similarity index, and finger print Tanimoto based coefficient to get natural phytochemicals. PubChem is a user friendly, online comprehensive database having precomputed list of structurally similar compounds of several query compounds. Similar compounds or similar conformers are called as "neighbours" like 2D neighbours or 3D neighbours when searched by more than 80% similarity index (Bolton et al., 2011). Out of 47 query compounds, 191 compounds were retrived on the basis of more than 80% similarity filter and Tanimoto based coefficient. Further, 191 obtained compounds were subjected to molecular docking in Autodock Vina.

Successful molecular docking relies on selection of accurate query ligand structure in proper format from proper source. PPAR-γ protein structure is extensively studied as its 130 structures are submitted in protein data bank (PDB). The root mean square deviation values (RMSD) of different PPAR-γ protein structure is less than 0.5 Å (0.488±0.109 Å) (Encinar et al., 2015). The preference is mostly given to structures with lower resolution value, crystallized with ligand and with no mutated or missing residues. All kind of information about every protein ID is given in PDB. Mostly PDB entries consist of protein structure complexed with ligand, inhibitor or modulator (Josh et al., 2015). In present study, PPAR-γ protein structure complexed with its co- crystallized ligand cercosporamide derivative with PDB ID 3V9T was selected and downloaded from PDB. 2D structures of all 191 compounds were downloaded from PubChem and then the energy of these structures was minimized in software called Avogadro. Energy minimization is an important process before molecular docking and molecular dynamics as it reduces steric clashes and ensures appropriate geometry and relaxed structure. All ligand structures were docked against 3V9T PPAR-γ protein structure to assess extent of protein ligand interaction and best binding pose. Molecular docking studies screens out potent compounds of high dock score. It was observed that PPAR-γ possess active site amino acid residues as HIS323, CYS285, SER289, TYR473, PHE282, LEU469, MET364, LEU330, GLY284, TYR327, HIS449, LEU453, TYR327 and ILE 326 (Selvaraj et al., 2014). As observed in docking interaction results of silymarin, hesperidin and glabrol, these all compounds share amino acids present in active site of PPAR-γ and interact by binding with them and also form hydrogen bonds. It was reported by Encinar et al., 2015, that PPAR-γ gets co-crystalized with cercosporamide then latter acts like a partial PPAR-γ agonist. Its active site interaction amino

acid residues were SER289, HIS449, CYS285, GLY284, LYS364, LEU330, ILE281, ILE341, VAL339, LEU353 and MET334 tells that natural agonists and other drugs or compounds with drug like activities can bind in active site of PPAR-γ and modulate its activity for drug development. Amino acid residues shared by hesperidin on active site of PPAR- γ during docking are MET364, LYS367, VAL339, LEU255, GLU259, SER342, CYS285, ARG280, ILE341, ARG288, LEU330 and GLY284. Whereas amino acid residues shared by silymarin on active site of PPAR-γ during docking are MET364, ILE341, SER342, CYS285, SER289, PHE287, GLY284, ARG288, LEU330, LYS367 and amino acid residues shared by silymarin on active site of PPAR-γ during docking are PHE287, CYS285, ARG288, GLY284, ARG280, LYS367, ILE281, SER342, ILE341, LEU255 and LEU330. In obtained result of docking it can easily be seen that all the three compounds namely silymarin, hesperidin and glabrol have shared CYS285 and SER342 amino acid residues during docking upon PPAR-γ receptor. It has been reported that with minor exceptions, mostly partial PPAR-γ agonists interact with the ligand binding domain (LBD) of PPAR-γ receptor and binds on SER289 and SER342 amino acids residues forming hydrogen bonds, while rest of the interactions were found hydrophobic (Furukawa et al, 2012, Julius et al, 2018, Farce et al, 2009). Thus, based on these literatures, it is evident that all silymarin, hesperidin and glabrol are partial PPAR-γ agonists as they all interact with LBD of PPAR-γ receptor through SER289 and SER342amino acids residues formig hydrogen bonds, while rest of the interactions were found hydrophobic. Pferschy-Wenzig et al, 2014 identified isosilybin, a constituent of silymarin as PPAR-γ agonist by performing docking studies. Zhiguo L. et al, 2022 docked natural compounds to screen out PPAR-γ agonist. In that study they found hesperidin as a PPAR-γ agonist. Further, these three compounds were subjected to ADME studies.

For a compound to act like a drug, it must reach its target receptor in appropriate concentration and must stay there in proper concentration for proper duration to exhibit expected results (Daina et al., 2017). This drug like properties were evaluated by performing ADME studies in which 9 compounds of highest dockscores, obtained through molecular docking studies, were subjected to ADME studies through 5 different softwares namely Swiss ADME, admet SAR, ADMETlab, DruLiTo and vNN ADMET. These all softwares gave results in different parameters, so we took 17 common parameters to conclude result of ADME. As given in table 10, compound 1(silymarin), compound 4 (hesperidin) and compound 9 (glabrol) are obeying Lipinski rule, which says that Molecular weight (MW<500), H bond acceptor < 10, H bond donor < 5 and Log p< 5, if a drug obeys all these rules then it can be considered as lead

compound for drug development. Then other parameters like Total polar surface area (TPSA, which tells about polarity of compound) should be between 20-130. Compound 4 and 9 are obeying this condition although compound 1 is going slightly high, rest of the molecules are going highly out of range. Then bioavailability score is again optimum for compound 1, 4 and 9 only, rest of the compounds are having lesser one. Log S, i.e. water solubility should be greater than -4, which is being followed by all the compounds. Next parameter is log D, which is a log of partition of a chemical compound between the lipid and aqueous phases i.e. Log D tells that compound would be more hydrophilic or lipophilic. More negative LogD, less lipophilic compound so poormembrane permeability, so for better membrane permeability, value of log D should be less –ve, which is observed for compound 4, 5, 7 and 9. Then, another parameter is rotatable bond, which is measure of molecular flexibility and is important in assessing oral bioavailability of drug, hence it should be less than 10 for good bioavailability of drug, which is observed for compounds except compound 7. Then molar refractivity (MR), which is molar volume corrected by refractive index. it represents size and polarizability of a fragment or molecule. It should be between 40 to 130, which is obeyed by compound 1, 4 and 9 only. Then next parameter is log Kp (skin permeation), i.e. measure of skin to absorb any compound, more –ve value of log Kp, less would be the skin permeation, again compound 1, 4 and 9 are best among others as they have fewer negative values so more would be the skin permeation of drug. Synthetic accessibility means how much it would be easy to synthesize a drug. If the value is 1 then it would be very easy to synthesize a drug whereas if the value is 10 then it would be very difficult to synthesize a drug. For this also, again the value of compound 1, 4 and 9 is best among other compound astheir value is closer to 1. PAINS (PAN Assay interference compound) Alerts, tells about the presence of particular fragment in drug which will always show positive result, no matter which target is being taken, that means it shows false positive result. It is 0 in all molecules except molecule 8. BRENK Alerts shows the presence of any particular fragment in drug which couldbe toxic, reactive and unstable etc., its value is also found 0 for all compounds except 7, 8 and 9. That means all compounds except 7, 8 and 9 do not have any toxic, reactive and unstable fragment. Then, another parameter, PPAR-γ binding, that means ability to bind with PPAR-γ receptor, is also good for 4, 7, 8 and 9. Then, in last P-glycoprotein, which excretes xenobiotics (drug or toxin) outside the body and induces multi drug resistance. Hence cancer cells usually increase number of p-glycoprotein for developing multi drug resistance. So, a good drug should be p- glycoprotein inhibitor. So, value of p-glycoprotein inhibitor should be more then it will bea good drug, its value is found highest for molecule 1 in comparison to others. Rathaur P. et al, 2021 performed ADME studies of

silybinin, a constituent of silymarin against TNBC. They observed decrease in the IC_{50} value of paclitaxel with the silibinin along with other tested natural compounds. Rest of the two compounds hesperidin andglabrol were not studied before forTNBC by ADME study. From ADMET result analysis, 3 compounds namely Silymarin, Hesperidin and Glabrolhaving best drug like characteristics were obtained, which were later subjected to MDS.

Molecular dynamics simulates motion of particles of system, when applied to biological systems exhibits fluctuation of relative position of atoms inside the protein as a function of time. This knowledge gives insights of biological phenomenon (Karplus, M. et al, 1990). This present molecular dynamics study ultimately revealed binding free energy for each protein ligand complex. The binding free energy of ligands (Silymarin, Hesperidinand Glabrol) complexed with protein PPAR-γ receptor was calculated in Prime tool of Schrodinger package from last 100ns. It was found that Hesperidin was having highest binding free energy (-85.958 kcal/mol) as compared to co-crystallized ligand (-82.951 kcal/mol). However, other molecules Silymarinand Glabrol were having less binding free energy (-72.036 kcal/mol and 71.054 kcal/mol, respectively) as compared to crystallized ligand i.e. cercosporamide. The Overall MDS results reflected that Hesperidin was found as best compound against PPAR-gamma protein as an agonist. This could be potential lead against PPAR-gamma but need to be proven experimentally. The overall results of these studies, found that Hesperidin had higher binding potential against PPAR-gamma as compared to Silymarin and Glabrol. However, all the compounds were found stable during the RMSD, RMSF and radiation of gyration studies. In other study, silibinin which is an active component of silymarin was evaluated along with paclitaxel to assess the anticancer potential against metastatic breast cancer and observed decrease in the IC_{50} value of Paclitaxel along with silibinin but they didn't performed study against PPAR-γ receptor (Rathaur, P. et al, 2021). Madureira, M. B. et al, 2023 performed a molecular simulation study to showthat hesperidin acts as promising co-adjuvant therapy for prevention of TNBC but they didn't consider PPAR-γ in study. However, there is not even a single study revealing anticancer potential of glabrol through molecular dynamics simulation studies for TNBC yet. Our study first time reported simulation of the binding of these three compounds namely hesperidin, silymarin and glabrol against PPAR-gamma as an agonist to understand their molecular mechanism and to depict the important sight points for binding onto PPAR-γ receptor to ameliorate TNBC. Among all these 3 compounds i.e. hesperidin, silymarin and glabrol, we chose hesperidin and silymarin for invitro studies, as glabrol was good but it wasvery expensive so we didn't consider it for further studies.

***Invitro* study:**

In *invitro* study, different types of assays were done on test compounds namely silymarin and hesperidin against standard drug compound doxorubicin to assess the anticancer potential of test compounds. We conducted six types of invitro assays namely DPPH free radical scavenging assay, Cell viability assay, PPAR-γ agonistic activity assay, Antioxidant enzyme activity test, Western blotting and Real time PCR.

Reactive oxygen species (ROS) or free radical geerated during cellular processes damages lipids, nucleic acids and proteis inside our body leading to altering their function, thus it must be rectified by antioxidative defense system. When balance between ROS production and antioxidative defense system is disturbed then codition of oxidative stress is generated inside the body (Jelic, M. D. et al 2021). Oxidative stress is a major cause of different kind of diseases including cancer. Various exogenous factors like ultraviolet rays etc and endogenous factors like cellular reactions are involved in increasing oxidative stress in human body. When oxidation- reduction system of human body gets weakened then oxidative stress increases more than capacity of oxidation reduction system which further leads to carcinogenesis (Noda N. et al, 2001). This oxidative stress must be decreased by scavenging free radicals generated in ourbody. This free radical scavenging is done by antioxidants. Antioxidants are natural compounds present in food that neutralize and scavenge free radicals. Antioxidants basically give their own electrons to free radicals for neutralizing them. Foods rich in antioxidants neutralize free radicals and thus reduce their impact in human body. Therefore, it is essential to explore natural phytochemicals for their antioxidant potential (Rahman et al., 2015). DPPH free radical scavenging assay measures the antioxidant potential of test compounds. We performed DPPH assay for assessing antioxidant potential of silymarin and hesperidin. DPPH assay was performedby the method explained by Sharma et al., 2009. Results of this assay indicate that rise in % inhibition of the free radical DPPH takes place in a dose dependent manner with the increasing concentration (50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml) of silymarin and hesperidin revealed that both the phytochemicals had antioxidant potential. Ascorbic acid was taken as a standard in this assay. IC_{50} of ascorbic acid was 2.83196235 whereas IC_{50} of silymarin and hesperidin was observed as 30.35991 and 53.171041 respectively. Thus, silymarin found to be better antioxidant than hesperidin in this assay as shown by its lower IC_{50} value. As IC50 of Silymarin is lower than Hesperidin, so Silymarin will be considered of higher antioxidant potential than Hesperidin. As the values of % DPPH

inhibition by both compounds are less than 0.05 level of significance, so, results are statistically significant.

In other study, DPPH assay was performed by Tong S. et al, 2011 for quantifying antioxidant potential of silymarin, 2,3-dehydrosilymarin, silybin, 2,3-dehydrosilybin and 2,3-dehydrosilycristin. The rise in % inhibition of the free radical DPPH takes place in a dose dependent manner. The IC_{50} for each compound was calculated. The obtained IC_{50} values of silybin, 2,3- dehydrosilybin, silymarin, 2,3-dehydrosilymarin and 2,3-dehydrosilycristin were 803.93 μ g/ml, 23.28 μg/ml, 24.67 μg/ml, 16.30 μg/ml and 18.43 μg/ml, respectively. Thus results indicate that 2, 3-dehydrosilybin, silymarin, 2, 3-dehydrosilymarin and 2, 3-dehydrosilycristin had better antioxidant potential than silybin. Even 2, 3-dehydrosilymarin had better free radical scavenging capacity than silymarin as revealed by its lower IC_{50} value. LIU, Y. et al, 2015 conducted a DPPH study to assess the potential of hesperidin, limonene and tea polyphenols for their antioxidant potential. Results of this assay indicate that rise in % inhibition of the free radical DPPH takes place in a dose dependent mannerwith the increasing concentration. The antioxidant potential of hesperidin(IC_{50}=0.097) is better than the limonene (IC_{50}=0.00236), tea polyphenol (IC_{50}=0.0144). Thus, the antioxidant potential of hesperidin is more than tea polyphenol and limonene in vitro. Thus, it is evident from these studies that both silymarin and hesperidin have good antioxidant capacity.

Both silymarin and hesperidin were also evaluated on cell line MDA MB-231 to check deleterious effect if any on treatment with test compounds by assessing cell viabilityin preclinicalstudies. MTT assay evaluates capacity of test componds to kill cancer cells by assessing cell viability, it also measures cell cytotoxicity to know whether a test compound is toxic for cells or not. It is based on the fact that metabolically active live cell will release succinate dehydrogenase which reduces MTT into formazan crystals (Vistica et al., 1991). It is used to know cell proliferation, cell viability and cell cytotoxicity (Mosdam, 1983; Denizot & Lang, 1986; Hansen et al., 1989). In present study, we checked cell viability by performing MTT assay on MDA MB- 231 cell lines using different concentrations of silymarin, for all hesperidin and standard drug doxorubicin. IC_{50} of silymarin was found to be 2.47 μM, while IC_{50} of hesperidin and doxorubicin was found to be 26.85 μM and 1.11 μM respectively. Thus, Silymarin is although less potent than doxorubicin but still more effective than hesperidin as it shows better cytotoxic activity against cancer cell. Hesperidin showed weaker cytotoxic activity or anticancer activity than Silymarin and doxorubicin. As the p values of MTT assay for both compounds are less than 0.001 level of sigificance, so, results are statistically significant. We

observed that there is dose dependant continuous decrease in cell viability of MDA MB-231 cell line for all the compounds at concentration 3µM, 6µM, 12µM, 24µM. For hesperidin, the cell viability was atleast 60% at concentration of 24µM. However, in case of silymarin the cell viability got reduced to 35% at concentration of 24µM and in case of doxorubicin, the cell viability was reduced to 15 to 17% at concentration of 24µM. This result indicates that doxorubicin being a standard drug of cancer treatment reduces cell viability of TNBC cancer cells MDA MB-231 the most, after that silymarin was found to be better phytochemical drug candidate as it had reduced cell viability of TNBC cancer cells MDA MB- 231 more than hesperidin.

Amalina N. D. et al, 2023 reported previously that both doxorubicin and hesperidin separately had cytotoxic effect on several cancer cells. In this study, they evaluated combined cytotoxic effect of both hesperidin and doxorubicin on metastatic breast cancer cells or TNBC cells 4T1 byusing MTT assay. They analyzed cell cycle arrest and apoptosis using flow cytometry to understand mechanism of this cytotoxic effect. In MTT assay, on 4T1 cells hesperidin showed increased cytotoxic effect with increasing concentration having IC_{50} value 284 µM. Hesperidin synergistically increased cytotoxic effect of doxorubicin which is related with the increase in apoptosis, G2/M cell cycle arrest and stopped the migration of 4T1 cells.

Dariushnejad, H. et al, 2022 also assumed comination therapy of vinblastine and silibinin which is active component of silymarin to reduce the dose and toxicity of vinblastine. MDA MB-231 cells were used in MTT assay for combination of 30µMsilibinin and 4 µM vinblastine's IC50 was 0.69. This clearly shows that combination of vinblastin and silibinin reduces side effects of vinblastin.

We also performed MTT assay on L929 cells which are not cancerous cells to assess whethersilymarin and hesperidinare decreasing viability of cells upto treatment or not. So, we found in our experiment that in case of silymarin % cell viability decreases upto 20 % at the concentration of 50µg/ml. However, in case of hesperidin %cell viability decreases upto 17% at the concentration of 50µg/ml. This shows that silymarin kills less normal cells than hesperidin. Thus, overall in MTT assay silymarin found to be better than hesperidin. So, in this cell viability assay on MDA-MB 231 cells we obtained IC50 with doxorubicin 1.11 µM, silymarin 2.47 µM and with hesperidin 26.85 µM whereas on L929 cells we obtained IC50 with doxorubicin 10.33, hesperidin 26.01 and with silymarin 17.45. So, it is evident from this result that compound hesperidin is better in decreasing cell viability in MDA-MB 231 cells than

silymarin due to lower IC50 value. Here, doxorubicin being standard compound is most toxic to L929 cells. As a chemotherapeutic agent, doxorubicin causes significant cell death even at low concentration. Its potency here reflects strong toxicity to normal cells. However, IC50 of Silymarin is more than doxorubicin, so it is moderately cytotoxic or less toxic than doxorubicin indicating better biocompatibility. Whereas, Hesperidin having highest IC50 is least cytotoxic to L929 cells and is most biocompatible compound and least toxic to normal fibroblast cells. As the values of MTT assay by both compounds are less than 0.05 level of sigificance, so, results are statistically significant.

On the other hand, PPAR-γ agonistic activity of silymarin and hesperidin was measured in vitro byPPAR-γ transcription factor assay method. This assay was performed by using PPAR- γ trascriptio factor assay kit item no. 10006855 on MDA MB-231 cells. It was a non- linear dose dependant increase in % agonistic activity of silymarin and hesperidin with increasing concentration of PPAR-γ receptor and compared with pioglitazone and doxorubicin. As the values of p for PPAR-γ agonistic activity of different ligands of PPAR-γ at different concentrations are less than 0.01 level of sigificance, so, results are statistically significant. Here, hesperidin is found as better PPAR-γ agonist than silymarin as visible in graph.

Results showed that pioglitazone had highest level of PPAR-γ agonistic activity in comparision to control. After that, hesperidin got better PPAR-γ agonistic activity then silymarin and last there was doxorubicin as it is not a PPAR-γ agonist. It was reported by Wang et al, 2014 that mostly all the natural compoundswhich activate PPAR-γ are weak agonist of it, as natural compounds activate PPAR-γ receptor partially in comparision to full agonist of PPAR-γ and act as partial PPAR-γ agonists.

Further, total protein concentration was determined using standard method of Lowry et al, 1951, in which bovine serum albumin was used to draw standard curve. It is essential to estimate total protein content of any cell or tissue sample before proceeding to further analysis (Smith et al, 1985) as all enzymes are proteins and estimation of total protein content is required for further calculation of enzyme activity. Further, determination of specific activity of enzymatic estimation is essential for comparative and analytical purposes (Lowry et al, 1951).

Punnonen K. et al, 1994 analysed lipid peroxidation products andantioxidant enzyme activity in breast cancer tissues in comparision to reference tissue. They observed that catalase activity of breast cancer tissue was lower than reference tissue whereas, SOD, hexose moophosphate shunt and glutathione peroxidase were increased. However, TBA was decreased in breast

cancer tissue, but its level in serum was higher, but amount of conjugated double bonds were equal in both cancerous and reference tissue. Moreover, they did not find any difference between diene conjugation content of serum and peroxyl- radical scavenging capacity of breast cancer patients and healthy people. Thus, this study indicated that in breast cancer patients antioxidant defence system is deranged, but it does not support notion thatlipid peroxide formation is most important in carcinogenesis.

On the other hand, Kangari, P. et al, 2018 also determined oxidative stress, antioxidant status and lipid peroxidation level. They observed that in breast cancer patients MDA level and SOD activity were found higher significantly in comparision to normal subjects, whereas glutathione peroxidase activity got significantly decreased in breast cancer patients in comparision to normal healthy subjects. Thus they concluded that increased SOD activity is due to increased superoxideanion and high lipid peroxidation and oxidative stress is high risk factor in the development and progression of breast cancer.

Arzi, L et al, 2022revealed that silibinin, a component of silymarin repressesWnt/β-catenin signaling pathway and thus helps in the treatment of TNBC. Arab, F. L. et al, 2024 elucidated antioxidant, antiproliferative and antiapoptotic effects of nano micelle of silymarin on mesenchymal cells. Lambona, C. et al, 2024observed that usage of milk thistle which contains silymarin reduces oxidative stress and thus can treat TNBC too.

On the other hand, Nandakumar, N. et al, 2011 evaluated therapeutic potential of hesperidin by assessing antioxidant enzyme activity, lipid peroxidation, membrane ound marker enzymes etc. in kidney tissues of DMBA induced breast cancer rats. After oral administration of 30mg/kg (of body wt.) hesperidin daily, there was significant decrease in renal lipid peroxidation and membrane bound marker enzymes. However, remarkable increase in antioxidant effect was observed. Thus, they concluded that these protective effects of hesperidin can be used further for treating oxidative stress mediated diseases. Nandakumar, N., Jayaprakash, R. et al, 2011 observed that upon administration of hesperidin into DMBA induced breast cancer in rats, lipid peroxidation was attenuated whereas increases total protein content in liver, breast and serum suggesting that it maintains cell structure and integrity of cell which promotes the usage of hesperidin for treatment of breast cancer. Ahmadi, A. et al, 2016 summarized that hesperidin being a flavonone glycosides, found in citrus species has cancer chemopreventiveaction and antioxidant and anticancer role.Nabil, I. et al, 2024 observed thathesperin had a protective action on finasteride-induced oxidative stress, testicular structural damage and apoptosis.

In our study, we analyzed comparative antioxidant potential of silymarin, hesperidin and doxorubicin. We observed that upon administration of silymarin on MDA MB-231 cells, there was slight increase in GST activity of cells in comparision to control, while upon treatment of hesperidin there was significant increase in GST activity in MDA MB-231 cells. However, doxorubicin decreased GST activity in comparision to control being a synthetic chemical anticancer drug. Whereas silymarin and hesperidin both being natural, partial PPAR-γ agonist increased GST activity which will further reduce oxidative stress. In this assay however hesperidin found to be better antioxidant than silymarin. Hesperidin has highest GST activity, shows it has highest antioxidant properties among all compounds, with highest level of significance. As the values of p for silymarin, hesperidin and doxorubicin treatment in GST assay on MDA-MB 231 cell line are less than 0.01 level of sigificance, so, results are statistically significant. These statistically significant differences indicate that the observed increases in GST activity with hesperidin and silymarin are not due to random chance and reflect real biological effects. Hesperidin's significantly higher GST activity suggests it strongly enhances antioxidant defense mechanism in MDA-MB 231 cells, while doxorubin's significantly lower GST activity confirms its known role in inducing oxidative stress and depleting cellular defense enzymes. We also analyzed changes in lipid peroxidation upon administration of silymarin, hesperidin and doxorubicin on MDA MB-231 cells. We found that doxorubicin increases lipid peroxidation upto high extent as there is great increase in accumulation of MDA. This is so, because doxorubicin is a synthetic chemical anticancer standard drug so it increases lipid peroxidation. However, silymarin and hesperidin both drecreased lipid peroxidation upto great extent at increased concentration i.e. 12µM, but hesperidin decreases lipid peroxidation even more than silymarin at 12µM concentration. Thus, it can be said that hesperidin is found to be better antioxidant than

silymarin. Doxorubicin, a chemotherapeutic agent is well kow to generate reactive oxygen species (ROS) and cause oxidative stress as a side effect. This result in higher lipid peroxidation, explaining its high value in this assay. On the other hand, Silymarin and hesperidin are natural antioxidants. They scavenge free radicals and protect membranes from oxidative damage, resulting in lower lipid peroxidation. As the values of p for silymarin, hesperidin and doxorubicin treatment in LPO assay on MDA-MB 231 cell line are less than 0.01 level of sigificance, so, results are statistically significant. As the results are statistically significant, so it can be said that observed results are not due to chance, but establishes prooxidant effect of doxorubicin while protective role of silymarin and hesperidin in reducing oxidative stress.

Catalase also is an antioxidant enzyme which decreases oxidative stress created by increase in concentration of hydrogen peroxide. Catalase activity was also measured in our study after administering silymarin, hesperidin and doxorubicin in MDA MB-231 cells. We found that doxorubicin treatment decreases catalase activity more at increased concentration i.e. 12µM as it is a synthetic chemical anticancer drug, so increases oxidative stress. However, silymarin and hesperidin both increased catalase activity at increased concentration i.e. more catalase activity at 12µM than 6 µM. Hesperidin increases more catalase activity than silymarin. So, hesperidin again found to be better antioxidant than silymarin.

Doxorubicin generates excessive free radicals but it reduces catalase activity due to doxorubicin induced oxidative stress which overwhelms antioxidant system, inhibiting catalase enzyme. Suppression of catalase shows failure of antioxidant defense under high oxidative stress caused by doxorubicin. Silymarin and hesperidin being natural compounds does not cause oxidative stress and increase catalase activity. Hesperidin has highest catalase activity, shows it has highest antioxidant properties among all compounds, with highest level of significance. As the values of p for silymarin, hesperidin treatment in catalase assay on MDA-MB 231 cell line are less than 0.01 level of sigificance, so, results are statistically significant. These statistically significant differences indicate that the observed increases in catalase activity with hesperidin and silymarin are not due to random chance and reflect real biological effects. Hesperidin’s significantly higher catalase activity suggests it strongly enhances antioxidant defense mechanism in MDA-MB 231 cells, while doxorubin’s significantly lower catalase activity confirms its known role in inducing oxidative stress and depleting cellular defense enzymes.

Then lastly in series of estimation of antioxidant activity, we estimated alteration in superoxide dismutase (SOD) activity upon administration of doxorubicin, silymarin and hesperidin. We observed that doxorubicin treatmentdecreases SOD activity too at increased concentration i.e. 12µM as it is a synthetic chemical anticancer drug, so increases oxidative stress. However, silymarin and hesperidin both increased catalase activity at increased concentration i.e. more catalase activity at 12µM than 6 µM. Hesperidin increases more catalase activity than silymarin. So, hesperidin again found to bebetter antioxidant than silymarin. In this result, hesperidin has highest SOD activity as it might be stimulating endogenous antioxidant defense mechanism, thus upregulatig SOD activity. Its antioxidant action could be partly indirect, promoting body's own antioxidant system, while doxorubicin being chemotherapy drug generates oxidative stress, so cells may compensate this stress by increasing SOD activity to some extent. So, observed increase in SOD activity may be cellular defense from free radical, not a direct benefit of doxorubicin. However, silymarin showing least SOD activity as it scavenges free radicals mainly by GSH and catalase, not always SOD. Low SOD can be due to dose dependent effect as low or high doses may not stimulate SOD optimally. Bioavalability, poor soluility or formulation issue can also be there. Silymarin showed less activity than doxorubicin due to differences in their chemical behavior and mechanism of antioxidant action. Quinone structure of doxorubicin participate in redox cycling, which can artificially enhance SOD like activity in some assays. However, silymarin works by enhancing endogenous antioxidant defense over time or chelating metal ions actionns that may not strongly affect the superoxide radical specifically in short term in vitro assay. It also lacks redox active groups like doxorubicin that might directly mimic or enhance SOD activity. Since p value is less than 0.001 for hesperidin, so result is statistically significant, that means higher SOD activity is not due to chance. So, in overall antioxidant enzyme activity estimation, hesperidin was found better antioxidant than silymarin.

Further, we also performed western blotting study to see the extent of expression of PPAR-γ receptor in MDA MB-231 cells upon administration of doxorubicin, silymarin, hesperidin and pioglitazone. We observed that pioglitazone increases PPAR-γ expression the most among all, as pioglitazone is a full, synthetic PPAR-γ agonist. However, doxorubicin which is a synthetic chemical anticancer drug and not a PPAR-γ agonist, so it does not trigger expression of PPAR-γ receptor much, but silymarin and hesperidin both triggered expression of PPAR-γ receptor, howeverresults reveal clearly that hesperidin increases PPAR-γ expression more than silymarin. Pioglitazone belongs to the thiazolidinedione (TZD) drugs acts as full agonist and

binds directly and completely to PPAR-γ and activates it fully. However, Silymarin being natural compound activates indirectly by reducing oxidative stress and inflammation, which in turn increase PPAR- γ expression. It does not bind completely as it is partial agonist thus its effect is weaker and slower, leading to moderate expression on western blot. On the other hand, doxorubicin, being a cytotoxic chemotherapy drug, does not increase PPAR-γ expression. In fact, it may reduce PPAR-γ expression due to induced oxidative stress. Thatswhy least PPAR-γ expression show by it. Results are statistically significant as p value is less than .01 for silymarin ad hesperidin and less than .001 for pioglitazone. This shows that these results are not due to chance.

Qu, Z., et al, 2020 observed that Brevilin A which is a natural compound a sesquiterpene lactone reduces growth of TNBC cells by affecting STAT3 and AKT/mTOR pathway. Protein bands were seen by utilizing ECL western blotting reagents for detection and expression of proteins was quantified by scanning densitometer and autoradiogram. Fatima I. et al, 2017 also performed western blotting study on natural compound Jatrophone which had antiproliferative action against TNBC cells by interfering Wnt/β catenin pathway.

Kim S.H. et al, 2021 performed western blot analysis on MDA MB-231 cells and MCF 7 cells for determining expression level of MAPK pathway protein. They investigated from this study that silymarin inhibits growth of breast cancer cells in humans by inducing apoptosis and by regulating MAPK pathway proteins. Kim S. et al, 2014 also used western blot technique to determine that silymarin, a product of milk thistle suppresses induction of fibronectin due to epidermal growth factor by the inhibition of STAT3 in TNBC cells.

Iqbal, M. A. et al, 2021 also conducted western blot technique to elucidate that silibinin, a component of silymarin creates metabolic crisis in TNBC cells by altering EGFR-MYC-TXIP axis. Byun, H. J., et al, 2017 found in western blot study that silibinin, a component of silymarin has antiproliferative effect by decreasing expression of MMP2 proteins by affecting Jak2/STAT3 pathway and also decreases metastatic potential of MDA MB-231 cells. Dariushnejad, H. et al, 2022 found in western blot technique that silibinin increases apoptosis mediated by vinblastine in TNBC cell line. In this process, BCL2/BAX and Caspase 3 pathway were also included.

Amalina, N. D. et al, 2023 found out in *invitro* condition using western blot analysis that hesperidin and doxorubicin synergistically down regulates transition of epithelial mesenchymal cell in TNBC or metastatic breast cancer cells. They showed that expression of protein Rac1 and MMP-9 proteinases increases in this process. Thus, they concluded that hesperidin along with doxorubicin can decrease the side effects of chemotherapy in treatment of metastatic breast cancer.

Zhao J. et al, 2017 also used western blot analysis and determined expression levels of cleaved protein caspase-3. This protein found involved in killing of ovarian cancer cell by involvement of hesperidin through endoplasmic reticulum stress signaling.

In the series of *invitro* studies, we also performed real time PCR to determine the expression of apoptotic, protooncogene or tumor suppressor gene like PPARG gene, BRCA1 gene, p53 gene, SDC1 gene, BCL2 gene and BAX geneafter treatment of MDA MB-231 cells with hesperidin and silymarin. We observed in this study that expression of PPARG gene, BRCA1 gene, p53 gene and SDC1 gene were upregulated when MDA MB-231 cells were treated with silymarin and hesperidin. However, expression of BCL2 gene and BAX gene was found down regulated after treating cells with silymarin and hesperidin. Both compounds silymarin and hesperidin enhance lipid metabolism, anti inflammatory response and tumor suppression. Upregulation of PPARG supports cell differentiation and inhibit tumor progression. Increased expression of BRCA1 shows increased genomic stability and protective anticancer effect. Increased expression of p53 shows both compounds may activate tumor suppressive pathways. Increased expression of SDC1 inndicate improved epithelial barrier integrity. Whereas downnregulation of BCL2 and BAX gene shows proapoptotic potential of both silymarin and hesperidin. This may be as intrinsic apoptosis is not dominant pathway being activated or that regulation is occurring post- trannscriptionally or is cell-type/time dependent. Genes like PPARG, BRCA1, p53 and SDC1 show significant upregulation, with p values less than 0.05, confirming that the treatments likely caused real, biological relevant changes, not by chance. BCL2 and BAX downregulation is also statistically significant, supporting its potential role in proapoptotic signaling. As the values of p for silymarin and hesperidin treatment in Real time PCR on MDA-MB 231 cell line are less than 0.05 level of sigificance, so, results are statistically significant.

Kongtawelert, P.et al, 2020 found out that hesperidin stops growth of cells of breast cancer by inhibiting expression of PD-L1 by downregulation of NF-kB and Akt signaling in TNBC. Khamis,

A. A., et al 2018 used real time PCR to determine the relative expression of different genes involved in causing cancer like Bax, Bcl2, EGFR and ERα genes. They concluded that hesperidin synergistically increases anticancer effect of tamoxifen for breast cancer. Patel P. et al 2021 showed in their experiment that hesperidin has protective effect against DMBA induced breast cancer in female rat by attenuating expression of Ki67. Feng, Y. et al, 2022 showed that hesperidin increases antitumor effect of carboplatin by inhibiting p53-MDMX interaction which induces apoptosis in non- small cell lung cancer.

Kim S. et al, 2016 also conducted real time PCR and found out that silibinin, the component of silymarin obtained from milk thistle plant, inhibits motility of TNBC cells by decreasing expression of TGF-β2. Iqbal M.A. et al 2021 observed in real time PCR that silibinin causes metabolic crisis in TNBC cells by altering axis of EGFR-MYC-TXNIP. Kil, W. H. et al, 2014 conducted real time PCR to show that silibinin being a polyphenolic flavonoid has anticancer effect on MDA MB-468 cells of breast cancer using xenograft model. Lashgarian, H. E. et al 2020 performed real time PCR amplification and showed that silibinin can stop migration of TNBC cells i.e. highly metastatic breast cancer by down regulating expression of RAC1 gene. Karimzadeh, M. R et al 2024 also conducted real time PCR to determine the expression of some genes after treatment of silymarin along with cisplatin, the standard chemotherapy drug which together inhibit growth of cells and induce apoptosis in ovarian cancer. Upadhyay, P. et al 2020 found that silymarin induces apoptosis in liver cancer when given in form of nanoparticle loaded in conjugation with lactobionic acid. Thus, it is proved from these *invitro*experiments that both silymarin and hesperidin have good anticancer property against TNBC.

***Invivo* study:**

Further, *invivo* study was conducted on eggs as invivo model. We studied alteration in angiogenesis upon treatment of fertilized chicken eggs with silymarin and hesperidin in comparision with standard chemotherapy drug doxorubicin as positive control in CAM assay. CAM assay has been used earlier as an invivo model for studying angiogenesis. In CAM assay we found that with negative control, no treatment was given to fertilized chicken eggs. So, there was large amount of angiogenesis in eggs. Basically, angiogenesis is essential for cancer cells

to survive as increase in formation of blood vessels in cancer cells helps in providing nutrition and oxygen to growing and metastasizing cancer cells. If angiogenesis will stop then proliferating and metastasizing cancer cells will be short off of nutrition and oxygen and finally will die. So, researchers around the globe use to search for compounds having capability to decrease angiogenesis. Thus, ability to decrease angiogenesis is considered as property of anticancer acompounds or drugs. Therefore, with same intention, we also used silymarin and hesperidin in CAM assay to check their capability to decrease angiogenesis in comparision to positive control standard chemotherapy drug doxorubicin.

However, when we treated fertilized chicken egg with the standard drug doxorubicin then there was complete inhibition of blood vessel formation or angiogenesis as doxorubicin is a chemical synthetic chemotherapeutic standard drug so it completely inhibits angiogenesis, but it causes several serious side effects, due to which usage of doxorubicin should not be promoted, instead we should search for natural products which have potential anticancer properties.

Thus, with this motive in mind, we performed CAM assay in fertilized chicken eggs with two different concentrations of silymarin and hesperidin. We found in CAM assay with silymarin that it reduces very less angiogenesis with concentration 3µg. However, when concentration of silymarin was increased from 3µg to 6 µg, then then inhibition was increased upto great extent, which was actually desirable for silymarin to have anticancer property.

Similarly, when we performed CAM assay with the two concentrations of hesperidin then we found thatat concentration 3µg reduction in angiogenesis was far more than silymarin at same concentration. However, at concentration 6 µg, there was complete inhibition of angiogenesis, which was not the case with silymarin at concentration 6 µg. So, it can be concluded from this CAM assay results that hesperidin was found as better drug compound with more antiangiogenic and anticancer capacity than silymarin. In CAM assay, it is observed that number of branches of blood vessels formed by different concentrations of test compounds silymarin and hesperidin is decreased. This shows that both compounds are potent antiangiogenic compounds. However, hesperidin was found much better than silymarin in efficiency as it completely reduced blood vessel formation at higher concentration. Negative control showed 0% inhibition however positive control doxorubicin showed 100% inhibition. Silymarin at concentration 3 µg showed less than 40% inhibition while at 6 µg inhibitionwas 60%. However, with hesperidin at 3 µg concentration inhibition was 90% and at 6 µg concentration inhibition was 100% like positive control doxorubicin. This is shown in fig 6.33. Hesperidin demonstrates strong inhibitory potential, possibly rivaling doxorubicin, while silymarin shows moderate, dose-dependent activity. These results suggest hesperidin may be a more promising candidate for further

development, especially considering its high efficacy at low concentrations. Decrease in the length of blood vessels formed on treatment with silymarin, hesperidin and doxorubicin is statistically significant w.r.t. control as p value is less than 0.001. Moreover, Hesperidin is significantly more potent than silymarin at the same concentration as the p value is less than 0.001. Silymarin shows dose-dependent, but less potent effects. These statistically significant differences support the conclusion that hesperidin is a more effective inhibitor of angiogenesis than silymarin under the tested conditions. As the values of p for silymarin, hesperidin and doxorubicin treatment in CAM angiogenesis assay on MDA-MB 231 cell line are less than 0.001 level of sigificance, so, results are statistically significant.

Yang, S. H. et al 2005 observed effect of silymarin in CAM assay that with dose dependant increase it inhibits angiogenesis by upregulating Flt-1 receptor but not KDR receptor. Agarwal, R. et al 2006 described that silymarin has anticancer potential having antiangiogenic effect in chicken CAM assay. Upadhyay, P. et al 2020 conducted CAM assay in chicken egg for studying the antiangiogenic effect for assessing antitumor effect of lactobionic acid conjugated PLGA nanoparticles which were loaded with silymarin. They found that it induces apoptosis in liver cancer cells. However, silymarin is not much explored compound with CAM assay for cancer and especially breast cancer and TNBC.

Hesperidin is also used to evaluate antiangiogenic potential in chicken CAM assay for ameliorating rheumatoid arthritis. They delivered nanocomposite hydrogel of hesperidin along with lenalidomide to assess immunomodulatory efficiency of lenalidomide and anti-inflammatoryand antiangiogenic capability of hesperidin in local joints affected by rheumatoid arthritis (Du, X. et al 2023).Yu, L. et al 2024 also used CAM assay as an invivo model to explore hesperidin as a prominent pharmaceutical excipient.Chauhan, R., et al 2022 also used CAM assay as an invivo model for assessing the antiangiogenic anti-inflammatory activity of hesperidin in nanoparticle form. They basically evaluated efficiency of hesperidin for genes like VEGF, COX2 and CD105 etc involved in tumor formation. Al-Rikabi et al 2020also used hesperidin in invivo model to evaluate its efficacy for inflammation and cytotoxicity. Rehman, U. et al 2024 used HET CAM assay for evaluating hesperidin loaded cubogelas a novel therapy for wound healing. So, It is clear that hesperidin has been explored in CAM assay for various types of diseases as an invivo model for antiangiogenic property also along with anti-inflammatory and therapeutic properties, but it is still very less or not at all explore for breast cancer especially TNBC. So, our study is a novel work exploring anticancer and antiangiogenic

potential of both silymarin and hesperidin against TNBC utilizing CAM assay as an invivo model.

As hesperidin was found more potent than silymarin in CAM assay, therefore we further performed cell invasion assay with hesperidin only just to assess the potential of hesperidin to inhibit the invasion of MDA MB-231 cells in germ layers of fertilized chicken egg. Inhibition potential of hesperidin establishes hesperidin as antimetastatic natural compound which will not have any serious side effects, the most urgent and important urge of research worldwide relatedto TNBC. Metastasis is a most crucial and alarming problem of TNBC as it leads to death of patients because cancer cells reaches to various perts of patient body, making possibility of treatment almost impossible. So, it must be prevented in some way. Chemotherapy drugs like doxorubicin although prevent metastasis, but side effects it causes worsens the life of patients. So, this problem needs to be addressed with utmost importance. As nowadays neutraceuticals or herbal or phytocompounds are promising therapeutic agents due to their less or no side effects sowe also used hesperidin, a phytocompound in cell invasion assay to assess its antimetastatic potential using MDA MB-231 cells in chicken egg. So, basically the capacity of hesperidin to inhibit the movement of MDA MB-231 cells in germ layers of chicken egg was assessed.

Poomipark, N. et al 2023 observed anti-invasion, antimigration and antiproliferative activity of hesperidin glycoside in non small cell lung cancer using A549 cells.Tan, S. et al 2020 also found that administration of hesperidin can suppress proliferation of cell in lung cancer as it targets miR- 132/ZEB2 signnaling pathway and promotes apoptosis too. Kongtawelert, P. et al 2020reported that hesperidin inhibits the expression of programmed death ligand (PD-L1) in breast cancer. Lee,
K. H. et al 2010 observed the effect of hesperidin to inhibit the invasivenessof tumor cell in liver cancer. This takes place by suppressing nuclear facor kappa B and activator protein 1. Ning, L. et al 2020found anticancer effect of hesperidin against prostate cancer cells inhuman by causing ROS mediated necrosis. They observed that use of hesperidin also reduced invasion of cancer cells substantially. Zhao, J. et al 2017 found that hesperidin inhibits viability of cells of ovarian cancer and also induces apoptosis by affecting endoplasmic reticulum pathway. Xia RongMu, X. R., et al 2018 found in trannswell assay that hesperidin inhibits invasion and migration of non small cell lung cancer by stopping SDF-1/CXCR-4 pathway. Pavan, S. R. et al 2024concluded that hesperidin in nanoformulation causes anticancer activity against lung

cancer by targeting MEK/ERK and Akt/mTOR pathways together and thus itinhibits cellular invasion and causes reduced expression of E-cadherin.

So, from these literature reviews and observed *insilico, invitro* and *invivo* results, it can be said that both hesperidin and silymarin had proved to act as good anticancer compound, as in *insilico* studies both silymarin and hesperidin had got good dockscore, better ADME profile than other selected compounds as they both obeyed Lipinski rule, good bioavailability, good logD, logS and other parameters too. However, in molecular dynamics study, Hesperidin proved to be better than silymarin, as Hesperidin was having highest binding free energy (-85.958 kcal/mol) as compared to co-crystallized ligand (-82.951 kcal/mol). However, other molecules Silymarinand Glabrol were having less binding free energy (-72.036 kcal/mol and 71.054 kcal/mol, respectively) as compared to crystallized ligand i.e. cercosporamide. The Overall MDS results reflected that Hesperidin was found as best compound against PPAR-gamma protein as an agonist. This could be potential lead against PPAR-gamma but need to be proven experimentally. To prove this experimentally, different *invitro* assays were carried out using silymarin and hesperidin to fnd out the better one. In DPPH assay, silymarin showed better result than hesperidin with higher % DPPH inhibition value and lower IC_{50} value than hesperidin. Then, again in MTT assay, silymarin was found to be better than hesperidin as in MTT assay, dose dependant continuous decrease in cell viability of MDA MB-231 cell line for all the compounds at concentration 3µM, 6µM, 12µM, 24µM was observed. For hesperidin, the cell viability was atleast 60% at concentration of 24µM. However, in case of silymarin the cell viability got reduced to 35% at concentration of 24µM and in case of doxorubicin, the cell viability was reduced to 15 to 17% at concentration of 24µM. This result indicates that doxorubicin being a standard drug of cancer treatment reduces cell viability of TNBC cancer cells MDA MB-231 the most, after that silymarin was found to be better phytochemical drug candidate as it had reduced cell viability of TNBC cancer cells MDA MB- 231 more than hesperidin. So, in this cell viability assay on MDA-MB 231 cells we obtained IC50 with doxorubicin 1.11 µM, silymarin 2.47 µM and with hesperidin 26.85 µM whereas on L929 cells we obtained IC50 with doxorubicin 10.33, hesperidin 26.01 and with silymarin 17.45. So, it is evident from this result that compound silymarin is better in decreasing cell viability in MDA-MB 231 cells than hesperidin due to lower IC50 value. However, on L929 cells as IC50 of hesperidin is higher than silymarin it shows that it would be less toxic than silymarin on normal cells, which is very much needed quality as a drug. So again hesperidin proved to be a better compound than silymarin. Further in antioxidant enzyme activity, hesperidin proved to be

better than silymarin in GST, catalase assay, lipid peroxidation and SOD assay, as hesperidin increased enzyme activity more than silymarin. Further, in western blotting, again hesperidin increased more PPAR-γ expression than silymarin, however pioglitazone increased highest expression of PPAR-γ being its full agonist. Whereas hesperidin being partial agonist increased PPAR-γ expression more than silymarin. Then, further in Real time PCR, again hesperidin and silymarin both increased expression of PPARG, SDC1, p53 and BRCA1, gene having anticancer potential but decreased BCL2 and BAX. This increase in gene expression was more for hesperidin. Then in transcription factor binding assay, hesperidin showed better binding capacity to PPAR-γ than silymarin, pioglitazone being highest as full agonist of PPAR-γ. Then further invivo studies were conducted on fertilized chicken eggs. In this CAM angiogenesis assay was performed, in which hesperidin showed lesser no. of blood vessel formation upon application than silymarin. % inhibition of angiogenesis by hesperidin was higher than silymarin, highest being doxorubicin being the positive control. So, further in cell invasion assay we took two different concentration of hesperidin only to check it against doxorubicin. We found that at lower concentration there was penetration of MDA MB-231 cells upto certain extent, however at higher concentration of hesperidin effect was similar to doxorubicin, that is no cell penetrated across the tissues, which shows that hesperidin has got very good antimetastatic capability similar to doxorubicin. So, keeping all these results in mind, we can conclude that hesperidin is better anticancer compound than silymarin against TNBC Hesperidin has been used against liver, lung, prostate and breast cancer, but it has never been used earlier to explore antimetastatic property against TNBC. So, our research is completely novel in this regard as it explores hesperidin regarding antimetastatic activity using cell invasion assay against TNBC. In cell invasion assay against TNBC performed on fertilized chicken eggs using MDA MB-231 cell lines, we found that hesperidin at 3µg decreased metastasis into the tissues of egg. However, at 6µg it completely inhibited metastasis like positive control doxorubicin. So, its extent of anticancer properties is good enough to consider it to act like a drug for TNBC.

CHAPTER 8

CONCLUSION

CONCLUSION

Cancer is a group of diseases, characterized by abnormal rapid cell growth, affects different vital body parts at the stage of metastasis and causes death. In breast cancer, breast cells grow abnormally and metastasize to other organ which is fatal. However, TNBC differs from other types of breast cancer as in TNBC cancer cells do not have receptors for estrogen, progesterone and Her2neu. Thus, TNBC patients can not be treated with hormonal treatment thus left with limited treatment options like surgery, chemotherapy, radiotherapy etc., which is associated with devastating side effects. So, we need to find some other treatment options which target some otherreceptor present in TNBC. One such expressed receptor in TNBC is PPAR-γ receptor. PPAR-γ is already a much explored receptor for diabetes. Although PPAR-γ receptor is found involved in ameliorating different types of cancers but it is still very less explored in case of TNBC. So, we targeted PPAR-γ receptor against TNBC in our research. PPAR-γ is mainly activated by its synthetic full agonists called thiazolidiediones, but these compounds pose serious side effects like cardiotoxicity, hepatotoxicity and joint problems etc. So, other natural PPAR-γ agonists need to be searched which can activate PPAR-γ receptor partially and causes less or no side effects. Thus, to search natural partial PPAR-γ agonist against TNBC, we devided our research into *insilico, invitro* and *invivo* methods.

Based on the literature review, we selected 47 natural PPAR-γ agonists as query sequence for data mining and similarity searching in Pubchem database and got 191 similar compounds which further subjected into molecular docking and 10 highest dockscore containing compounds (compared to protein docked ligand dockscore) were selected for further ADME studies which revealed compounds having better pharmacokinetic potential. From this study, we selected 3 compounds silymarin, hesperidin and glabrol for further molecular dynamics study, in which silymarin and hesperidin were found better and were selected for further *in vitro* studies, however hesperidin was found better than silymarin. Both silymarin and hesperidin showed excellent free radical scavenging capability in DPPH test in dose dependant manner thus revealing that both compounds have good antioxidant potential however silymarin was found to be better than hesperidin in comparision to standard ascorbic acid, as silymarin's IC_{50} was 31.45 and hesperidin's IC_{50} was 54.23 and IC_{50} of ascorbic acid was 8.69. We also assessed the toxicity of both silymarin and hesperidin on MDA MB-231 cells (human TNBC cell lines) and L929 cells (mouse skin cell lines) by performing cell viability assay (MTT assay). We found that both hesperidin and silymarin killed cancer cells whereas didn't kill normal cells

which showed that both the compounds are good drug candidates. Antioxidant enzyme activity of superoxide dismutase, catalase, lipid peroxidation and glutathione s transferase in MDA MB-231 cells due to the effect of both silymarin and hesperidin was determined. We found that both silymarin and hesperidin increased enzyme activity of mentioned enzymes as these compounds are natural compounds and decreased lipid peroxidation also in contrast with the results of doxorubicin which is a standard synthetic chemotherapy drug, increased lipid peroxidation and decreased antioxidant enzyme activity. However, hesperidin was found better in antioxidant action than silymarin. In transcription factor binding assay both the compounds were found as good agonist but hesperidin was found better than silymarin. In western blotting, both the compounds increased expression of PPAR-γ receptor but hesperidin was found more capable than silymarin. In real time PCR, expression of genes involved in preventing and causing cancer were studied. We found that PPAR-γ, BRCA1, p53 and SDC1, acting as tumor suppressor genes were upregulated whereas BAX and BCL2 genes were down regulated due to the effect of both compounds silymarin and hesperidin in TNBC cells MDA MB-231.

We performed invivo study on fertilized chicken eggs by performing two types of assays. First is CAM angiogenesis assay and second is cell invasion assay. In CAM angiogenesis assay, we found that both silymarin and hesperidin decreased blood vessel formation, but hesperidin was better than silymarin as it completely inhibited angiogenesis at higher concentration like doxorubicin. Thus, hesperidin was found more potent as anticancer compound in comparision to silymarin. So, we took hesperidin only, for further cell invasion assay. In cell invasion assay, we found that hesperidin at 3µg decreased invasion of MDA MB-231 cells in egg tissues, whereas at 6 µg it completely inhibited metastasis like positive control doxorubicin did, this shows that hesperidin has better antimetastatic properties.

So, from results obtained in *insilico, invitro* and *invivo* studies done, we can conclude that hesperidin leads in tumor suppressive properties over silymarin, as in *insilico* studies hesperidin had better RMSD, RMSF and radius of gyration value w.r.t. protein, than silymarin. Then, in *invitro* studies also hesperidin proved to be better in antioxidant properties, decreased more cell viability of cancer cells than silymarin. Hesperidin increased more expression of PPAR-γ receptor, BRCA1, p53 and SDC1 (acting as tumor suppressor genes were upregulated) whereas BAX and BCL2 genes (involved in tumorigenic activity) were more down regulated by hesperidin than silymarin. Transcription factor binding assay, also proved hesperidin to be better and strong agonist of PPAR- γ receptor than silymarin as it bound strongly than silymarin. Further, in *invivo* studies, it was observed that hesperidin has better antiangiogenic and

antimetastatic properties (essential for any compound to act as anticancer compound) than silymarin. So, in view of all these findings, it is evident that hesperidin is better compound than silymarin, with more potent anticancer properties and thus it is the most suitable compound to act as a therapeutic option against TNBC, among all studied compound in this research work.

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