REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
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"Design, Synthesis and Biological Evaluation of Some Heterocycles as Non-Steroidal Aromatase Inhibitors"

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ABSTRACT
Cancer remains a critical global health issue and is the second leading cause of death after cardiovascular diseases, accounting for 1 in 6 deaths globally. By 2050, cancer cases are projected to rise to 27 million, with 17.5 million deaths due to demographic shifts. Breast cancer stands out as the most common cancer among women, with a significant incidence rate reported across various regions, including India, where it constitutes 25-32% of all female cancers. Efforts to combat breast cancer have focused on the study of flavonoids, both natural and synthetic, which are known to inhibit the aromatase enzyme. This study involved designing 76 flavonoid derivatives, whose novelty was verified through databases like Sci-Finder and PubChem. Using Molecular Docking with Glide software, 40 compounds were selected for synthesis based on their docking scores. The synthesis involved chalcone derivatives, followed by ring cyclization, resulting in a 49-94% yield of flavonoid derivatives.
The synthesized compounds were characterized by spectroscopic techniques and tested for their antioxidant and cytotoxic properties. Out of 30 compounds tested for antioxidant activity, 18 were further examined for their effectiveness against breast cancer cell lines (MCF-7) through MTT assays. Compounds 6B, 2K, 4K, 6K, 4B, 2B, and 4C demonstrated superior cytotoxicity compared to the standard drug Letrozole. Six of these compounds were also assessed for aromatase inhibitory activity, with compounds 2B and 6B showing potent inhibition, outperforming Letrozole. These findings suggest that specific flavonoid derivatives hold promise as effective treatments against breast cancer, potentially surpassing current standards.

KEY WORDS: Breast cancer, Flavonoids, Aromatase inhibitors, Molecular docking, Synthesis, Antioxidant activity, Cytotoxicity, MCF-7 cell line.

INTRODUCTION

Cancer is a class of diseases in which a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues) and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors which are self-limiting and do not invade or metastasize. Most cancers form a tumor but some, like leukemia, do not. The branch of medicine concerned with the study, diagnosis, treatment and prevention of cancer is oncology. (1) Cancer is a major public health problem worldwide and is the second leading cause of death after cardio vascular disease and was responsible for 8.8 million deaths in 2015. Globally, nearly 1 in 6 deaths is due to cancer. Approximately 70% of deaths from cancer occur in low- and middle-income countries. (2) By 2050, the global burden is expected to grow to 27 million new cancer cases and 17.5 million cancer deaths simply due to the growth and aging of the population. (3) In economically developed countries the three most commonly diagnosed cancers are lung and bronchus, colorectal and prostate among men and breast among women. In economically developing countries the three most commonly diagnosed cancers are lung and bronchus, stomach and liver in men and breast and cervix of uterus in women. (2) In 2017, 1,688,780 new cancer cases and 600,920 cancer deaths are projected to occur in the United States. For all sites combined, the cancer incidence rate is 20% higher in men than in women, while the cancer death rate is 40% higher. (4) As per global cancer fact sheet, breast cancer is most common types of cancer among the all other types of cancer in India and in globally, most common cancer in women both in more and less developed regions with slightly more cases in less developed (883,000 cases) than in more developed (794,000) regions Breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths) and while it is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total), it is now the second cause of cancer death in more developed regions (198,000 deaths, 15.4%) after lung cancer. (5) (Fig. 1.1)

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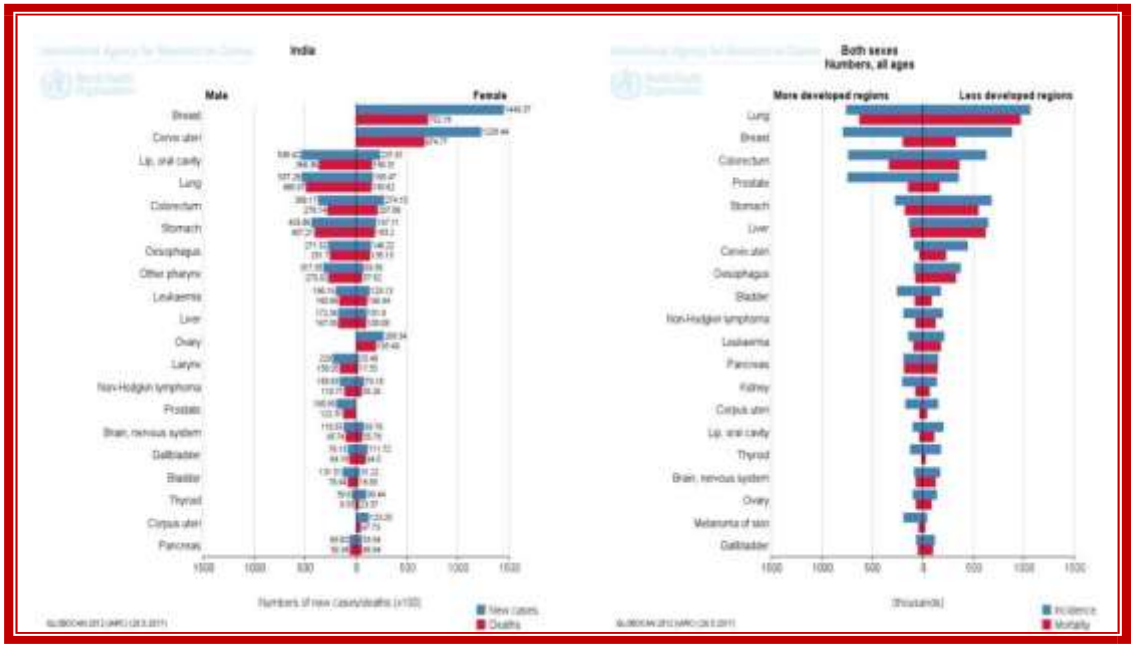

Fig.1.1 Cancer Statistics of India and Worldwide

Cancers are classified by the type of cells that resemble the tumor and therefore, the tissue is presumed to be the origin of the tumor. These are classified on the basis of the histology and the location of the cancerous cells. (6) (Table 1.1)

Table 1.1 Cancer Cell Classification Based on their Location and Histology

Types	Properties
Carcinoma	Malignant tumors derived from epithelial cells
Sarcoma	Malignant tumors derived from connective tissue or mesenchymal cells.
Lymphoma and leukemia	Malignancies derived from hematopoietic (blood-forming) cells
Germ cell tumor	Germ cell tumors are malignant (cancerous) or benign (non- cancerous) tumors that are comprised mostly of germ cells.
Blastic tumor or blastoma	This is a tumor (usually malignant) which resembles an immature or embryonic tissue.

TYPES OF TUMORS

Malignant Tumors (cancers) are usually named using -carcinoma, - sarcoma or -blastoma as a suffix, with the Latin or Greek word for the organ of origin as the root. Examples are Hepatic Carcinoma - a cancer of the liver, liposarcoma - a cancer of the fat. The most common type of breast cancer is called ductal carcinoma of the breast or mammary ductal carcinoma.

Benign Tumors (which are not cancers) are named using -oma as a suffix with the organ name as the root. Examples are leiomyoma - a benign tumor of the smooth muscle of the uterus. Unfortunately, some cancers also use the -oma suffix, examples being melanoma and seminoma. (7, 8)

CAUSES OF CANCER

The main causes of cancer are discussed under the following categories (9, 10)

- Radiation
- Viruses
- Chemicals
- Tobacco
- Alcohol
- Diet
- Hereditary Risk Factors
- Genetics

- Family History

BREAST CANCER

Breast cancer is a malignant growth originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Cancers originating from ducts are known as ductal carcinomas (85- 90% of all cases); those originating from lobules are known as lobular carcinomas (8% of all cases). Less common are inflammatory breast cancer and Paget's disease of the nipple. There are many different types of breast cancers, with different stages (spread), aggressiveness and genetic makeup; survival varies greatly depending on many factors. (11)

RISK FACTORS OF BREAST CANCER

The primary risk factors that have been identified for breast cancer are (12, 13)

- Age: Breast cancer can occur at any age, but the chance of having breast cancer is higher in women of age 45 and older.
- Personal history of breast cancer: A woman who had breast cancer in one breast has an increased risk of getting cancer in her other breast.
- Family history: A woman's risk of breast cancer is higher if her mother, sister or daughter had breast cancer. The risk is higher if her family members got breast cancer before the age of 40. Having other relatives with breast cancer (in either her mother's or father's family) may also increase a woman's risk.
- Having had high-dose radiation therapy to the chest (for example, in the treatment of Hodgkin's disease), especially if this treatment occurred between the ages of 11 and 30.
- Having your first child after age 30 or never giving birth to a child.
- Early menstruation (before age 13) or late menopause (after age 55), both of which result in a woman menstruating over a longer period of time.
- Having inherited a mutation in breast cancer susceptibility genes such as BRCA1 or BRCA2.
- Being overweight or gaining weight after menopause.
- Taking postmenopausal hormones (or hormone replacement therapy) after menopause.
- Having more than 3 alcoholic drinks per day.
- Taking birth control pills: Women have a slightly elevated risk while they are taking the pills.
- Certain breast changes: Some women have cells in the breast that look abnormal under a microscope. Having certain types of abnormal cells (atypical hyperplasia and lobular carcinoma *in situ* [LCIS]) increases the risk of breast cancer.
- Race: Breast cancer is diagnosed more often in Caucasian women than Latina, Asian or African American women.

The biology of breast carcinoma is complex with multiple factors contributing to its initiation and progression. The biomarkers that play key role in breast cancer development are steroid receptors [estrogen receptor (ER), progesterone receptor (PgR), retinoic acid receptor (RAR-β)) and selected suppressor/susceptibility genes (p^{53}, BRCA1, BRCA2)]. Prolonged exposure to circulating estrogens increase risk of breast cancer by mutation in selected tumor suppressor genes (p^{53}, BRCA1, BRCA2) accounting for 25% of all breast cancers.(14,15) Approximately 70-80% of breast tumors are ER positive (ER+). There are two types of estrogen receptors, ER-α and ER-β. Estrogens are the main ligands for these receptors. Prolonged exposure to circulating estrogens contributes to increased incidences of breast cancer. Removal of estrogens via oophorectomy decreases the risk of breast carcinoma.(16) In postmenopausal women, large part of estrogens in the breast tissue are derived from *in situ* biosynthesis using androgens as a substrate. Major sites of estrogen production are ovaries in premenopausal women, placenta in pregnant women and peripheral tissues such as fat, muscles and breast tissues in postmenopausal women. (17)

ESTROGENS

Estrogens are extremely important regulators of many physiological processes including maintenance of the female sexual organs, the reproductive cycle and numerous neuroendocrine functions. In breast cancer, through binding to their target receptor, they promote proliferation of breast cancer cells. (18) Estrogens enhance growth and proliferation of various target cells, such as breast epithelial cells and estrogen-dependent mammary carcinoma cells. (19) The binding of estrogens to receptor is the initial step in estrogen-mediated stimulation of DNA synthesis and cell replication in target tissue. (20)The beneficial effect of estrogens' depletion on breast cancer was initially noted in premenopausal women following oophorectomy. Since those early observation studies, the importance of estrogens as mitogens of breast cancer has been established in epidemiological, laboratory and clinical investigations. (21) Surgical approaches to estrogen-deprivation (ovariectomy, adrenalectomy and hypophysectomy) are efficacious, however, they lack specificity and are associated with significant side effects. (22)The etiology of breast cancer has a strong hormonal component. Once an epithelial cell of ductal system is transformed into a malignant phenotype, it is no longer subject to normal growth controlling mechanisms. A malignant cell may also be noninvasive, i.e. unable to penetrate the basement membrane (*in situ* cancer). Ductal carcinoma *in situ* is the most common histological variant of the non-invasive stage of breast cancer. Similarly, invasive or infiltrating duct carcinoma is the commonest form of breast cancer accounting

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for 85 to 90% of all cases. (23)

AROMATASE

Aromatase was isolated by Ryan in 1959 from the microsomal function of fresh human placental tissue.(24) Aromatase is the cytochrome P450 enzyme that converts androgens including androstenedione and testosterone to the estrogen products, estrone and estradiol, respectively.(25) The enzyme plays a key role in the regulation of these sex steroids. The aromatase gene, designated as CYP19, encodes the cytochrome P450arom and consists of 10 exons, with the exact size of the gene exceeding 70 kilobases. The gene is located on chromosome 15q21.1. The full length cDNA of 3.4 kilobases encodes for a protein of 503 amino acids with a molecular weight of approximately 55,000 daltons.(26) The amino acid sequence of P450arom is distinct from other members of the P450 cytochrome family and drugs have been developed with selectivity towards the cytochrome P450 moiety in aromatase, permitting more specific inhibition.
The enzyme complex is bound in the endoplasmic reticulum of the cell and is comprised of two major proteins. (27, 28)

1. *Cytochrome P450arom*: a hemoprotein that converts C19 steroids (androgens) into C18 steroids (estrogens) containing a phenolic A ring.
2. *NADPH-cytochrome P450 reductase*: which transfers reducing equivalents to cytochrome P450arom.

Whole aromatization of ring A of androgen, three moles of NADPH and three moles of oxygen are used in the conversion of one mole of substrate into one mole of estrogen product.(29,30)

AROMATASE INHIBITORS IN THE TREATMENT OF BREAST CANCER

Various estrogens can influence the risk of breast cancer and also the growth of established tumors. Hormone-dependent breast cancer tumors depend on estrogens for growth. Generally, there are two approaches treating these cases of breast cancer are either blocking the mechanism of action of estrogens (Antiestrogen) or inhibiting their synthesis (Aromatase inhibitors). A large number of aromatase inhibitors have been developed and utilized in clinical studies over the last 20 years. This development was prompted by the recognition of the fact that the cytochrome P450 inhibitor aminoglutethimide is an aromatase inhibitor (31).

MOLECULAR DOCKING

Molecular docking can be defined as an optimization process, which would describe the best fit orientation of a ligand into protein of interest. Docking in a true sense is the formation of non-covalent protein ligand complexes *in silico*. Conceptually, docking is an energy optimization process concerned with the search of the lowest free energy binding mode of a ligand within a protein binding site. During the course of the processes the ligand and the protein both adjust their confirmations to get the best fit and this kind of conformational adjustments resulting in the overall binding is referred to as “Induced fit”. The goal of ligand- protein docking is to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure. Successful docking methods search high-dimensional spaces effectively and use a scoring function that correctly ranks candidate docking (32). Docking constitutes two components (33): pose searching and scoring. Inclusion of protein flexibility is computationally expensive; therefore, much of the existing docking programs treat the protein either as rigid or allow flexibility only to the side chain functional groups. Ligand handling can be broadly classified as: whole molecule approach and fragment-based approach. A good docking method estimates the forces involved in the protein ligand recognition viz. electrostatic Van der Waals and hydrogen bonding and places the ligand appropriately in the active site. Docking can be used to perform virtual screening on large libraries of compounds, rank the results, and propose structural hypotheses of how the ligands inhibit the target, which is invaluable in lead optimization (34).

NATURALLY AVAILABLE FLAVONOIDS HAVING BREAST CANCER ACTIVITY

Chemistry of Flavonoids

It consists of a large group of poly-phenolic compounds having a benzo-γ-pyrone structure (Fig. 1.12) and present in the various plants having a wide variety of biological activities both *in-vitro* and *in-vivo*. Commonly, it is synthesized by phenyl propanoid pathway.

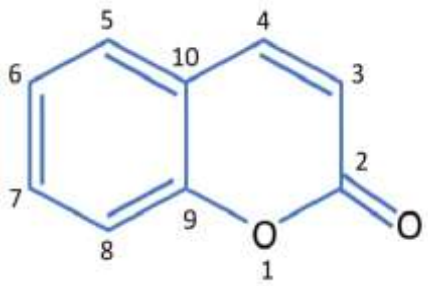

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Fig. 1.12 Structure of benzo-γ-pyrone

Chemically flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B as shown in Fig.1.13) linked via a heterocyclic pyrane ring (C)(35) They can be divided into a variety of classes such as flavones (e.g., flavone, apigenin, and luteolin), flavonols (e.g.,quercetin, kaempferol, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), and others. Their general structures are shown in Table 1.8.(36-37)

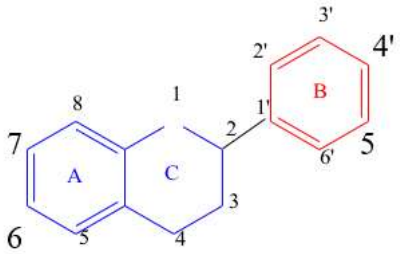
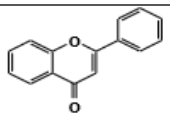
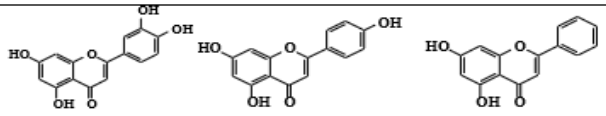
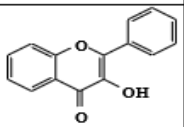
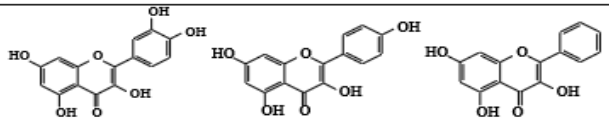
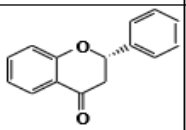
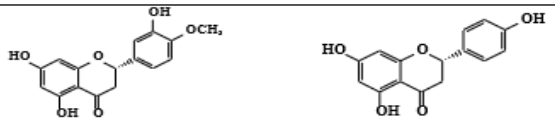
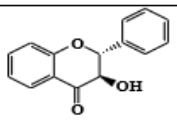
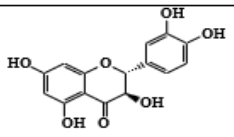
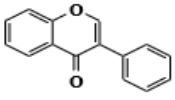
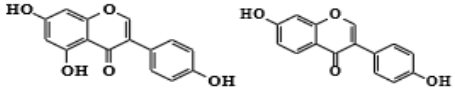
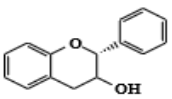
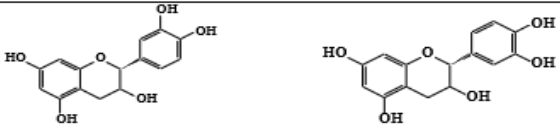


Fig. 1.13 Basic Structure of Flavonoids

The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings (35).

Table 1.8 Structure of Flavonoids (36)

Group of Flavanoid	Backbone Structure	Examples
Flavones		 Luteolin Apigenin Chrysin
Flavonols		 Quercetin Kaempferol Galangin
Flavanones		 Hesperetin Naringenin
Flavanonol		 Taxifolin
Isoflavones		 Genistein Daidzein
Flavan-3-ols		 Catechin Epicatechin

Flavonoids are group of plant phenolic compounds, occurring in all parts of plant, particularly the photo synthesizing plant cells. It is also coloring component of flowering plants. Flavonoids are an integral part of human and animal diet.

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Table 1.9 describe the food sources containing different classes of flavonoids.
Flavonoids in food are generally responsible for color, taste, prevention of fat oxidation, and protection of vitamins and enzymes.(38)

Table 1.9 Food Sources of Some Dietary Flavonoids2

Class	Flavonoids Presents	Dietary Sources
Flavanol	(+)-Catechin, (−)-Epicatechin Epigallocatechin	Tea
Flavone	Chrysin, apigenin Rutin, luteolin, and luteolin glucosides	Fruit skins, red wine, buckwheat, red pepper, and tomato skin
Flavonol	Kaempferol, quercetin, myricetin, and tamarixetin	Onion, red wine, olive oil, berries, and grapefruit
Flavanone	Naringin, naringenin, taxifolin, and hesperidin	Citrus fruits, grapefruits, lemons, and oranges
Isoflavone	Genistin, daidzin	Soyabean
Anthocyanidin	Apigenidin, cyanidin	Cherry, Rasberry, and Strawberry

Pharmacological Activities of Flavonoids
Numerous Flavonoids have shown various pharmacological activities which was isolated from plant sources like anti-microbial, anti-viral , anti-ulcerogenic , anti- oxidant, anti-hepatotoxic, antihypertensive, hypo-lipidemic, anti-platelet and anti- inflammatory activities(39)

Studies been have suggested that diet sources, including cruciferous vegetables, soy, rye flour, grapes, tea and mushrooms are associated with a decreased risk of breast cancer (40-44). In particular, due to their structural and functional similarities to endogenous estrogens, flavonoids have attracted considerable interest as alternative estrogens, termed phytoestrogens, and extensively studied for their potential role in many estrogen-dependent diseases including breast cancer. In fact, numerous flavonoids have shown interesting pharmacological activities in breast cancer biology, including binding affinities for estrogen receptors (45-47) anti-proliferative activities (48) and inhibitory activities against Aromatase enzyme. (49-51).

AIM AND OBJECTIVES:
AIM

- To design and synthesize flavonoid derivatives and assess aromatase inhibitory activity for the treatment of breast cancer

OBJECTIVES

- Design flavonoid derivatives by molecular docking studies.
- Predict ADMET properties (Drug Likeness) by using appropriate tools.
- Synthesize flavonoid derivatives and characterize by various pectroscopic techniques.
- Check the cytotoxicity of synthesized flavonoids on breast cancer cell-line (MCF-7) by MTT assay.
- Perform *In-Vitro* aromatase activity by fluorogenic kit method on selected compounds based on their docking score and results obtained from MTT assay.

MATERIALS AND METHODS
MATERIALS:
Materials Used for Synthesis

No	Name of Chemicals/Solvents	Procured From
1.	2′-Hydroxy-4′-methoxy acetophenone	TCI, Japan
2.	2′-Hydroxy acetophenone	Sigma Aldrich
3.	2′-Hydroxy-1′-acetonaphthone	Sigma Aldrich
4.	1′-Hydroxy-2′-acetonaphthone	Sigma Aldrich
5.	KOH Pellets	Merck
6.	Barium Hydroxide	Sigma Aldrich
7.	Lithium Hydroxide	Sigma Aldrich
8.	DMSO	Sigma Aldrich
9.	Methanol	Loba
10.	Ethanol	KUC, Chelthana

11.	HCl	Loba
12.	Iodine Granules	Sigma Aldrich
13.	Verataldehyde	SD Fine Chemicals
14.	3-Hydroxy Benzaldehyde	Sigma Aldrich
15.	3-Methoxy Benzaldehyde	Sigma Aldrich
16.	3-Chloro Benzaldehyde	Sigma Aldrich
17.	4- Chloro Benzaldehyde	Sigma Aldrich
18.	4- Fluoro Benzaldehyde	Sigma Aldrich
19.	4-Methoxy Benzaldehyde	Sigma Aldrich
20.	Cinnamaldehyde	Sigma Aldrich
21.	2,4,6 – Trimethoxy Benzaldehyde	Sigma Aldrich
22.	4- Pyridine carboxaldehyde	Sigma Aldrich
23.	3- Pyridine carboxaldehyde	Sigma Aldrich
24.	2- Pyridine carboxaldehyde	Sigma Aldrich
25.	2,3 – Dimethoxy Benzaldehyde	Sigma Aldrich
26.	2,4 – Dimethoxy Benzaldehyde	Sigma Aldrich
27.	4-Ethoxy 3-Methoxy Benzaldehyde	Sigma Aldrich
28.	3 – Fluoro Benzaldehyde	Sigma Aldrich
29.	Benzaldehyde	Loba

Materials Used for DPPH activity/MTT Assay/ *In vitro* Aromatase assay

No	**Name of Chemicals/Solvents**	**Procured From**
1.	2,2-Diphenyl-1-picrylhydrazyl	Sigma Aldrich
2.	MEM	Life Tech/Empire ent.
3.	MEM Non-Essential Amino Acids Solution (100X)	Life Tech
4.	Fetal Bovine Serum	Gibco
5.	Antibiotic-Antimycotic (100X)	Life Tech
6.	Trypsin-EDTA (0.25%), phenol red	Life Tech
7.	MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]	Hi-Media
8.	DMSO (For Molecular Biology)	Merck
9.	Isopropanol	SDFCL
10.	HEPES 1M solution	Gibco
11.	PBS 100X	Gibco
12.	*In-vitro* Aromatase Assay Kit (ElisaKit)	Bio-vision

List of Glass wares/Plastic wares

No	**Name of Chemicals/Solvents**	**Procured From**
1.	Round Bottom Flask- 20,50,100,150, 500 ml	Durasil
2.	Pipette – 0.1, 1,5 and 10 ml	Durasil
3.	Beaker – 10,25,50,100,150,250,500, 1000 ml	Durasil
4.	Dropping Funnel	Durasil
5.	Separating Funnel	Durasil
6.	Glass Stoppers	Durasil
7.	Calcium chloride guard tube	Durasil
8.	Condenser	Durasil
9.	Column	Durasil
10.	Volumetric Flasks – 10,50,100,500, 1000 ml	Durasil
11.	Magnetic beads	Durga
12.	Precoated TLC plates	Merck
13.	25 cm Narrow B.Vent Flask, Vented	BD
14.	50 ml tubes - Self Standing with Conical Bottom, Sterile	Axygen
15.	Microtest TC Plates - 96 Well Flat Bottom	BD

16.	Serological pipettes – 5ml	Tarsons
17.	Microtips (10 - 100 µl)	Tarsons
18.	Microtips (200 - 1000 µl)	Tarsons
19.	Microtips (0.2 - 10 µl)	Tarsons
20.	1.5 ml tubes	Tarsons
21.	0.2 ml Tubes	Tarsons
22.	0.22µm syringe filters	Axiva
23.	50 ml syringes (W/O needle) sterile	Nirlife Healthcare
24.	15 ml tubes	Tarsons

List of Instruments/Apparatus/Equipment's/Softwares used

No	**Name**	**Make/Model**
1.	Melting Point Apparatus	Veego, VMP-DS
2.	Heating Mantle	Durga Scientific
3.	Water bath	Dutt Enterprise
4.	UV Cabinet	Durga Scientific
5.	UV Visible Spectrophotometer	Shimadzu 1800
6.	IR spectrometer	Thermo Scientific, NICOLET 6700,
7.	Mass Spectrometer	Advion Compact Mass Spectrometer, ESI Technique
8.	^{1}H NMR spectrometer	400 MHz, Brüker Biospin, Switzerland
9.	Laminar Air Flow	Bioklenz TM
10.	CO2 incubator	New Brunswick, Galaxy Eppendorf, 170S
11.	Cooling Centrifuge	Eppendorf 5804R
12.	Fluorescent Microscope	Carl Zeiss, Axio Vert.A1
13.	Elisa Plate Reader	Epoch Microplate Spectrophotometer
14.	Elisa Plate Reader with Fluorescent Detector	BMG Fluostar (GERMANY)
15.	Maestro Suits 2016-1	Schrodinger
16.	Graph Pad Prism(ver.7)	Graph Pad Software, Inc
17.	Chemoffice 2004	Cambridge soft

Aromatase Reaction Preparation:
Prepare an Aromatase Substrate/NADP+(3X) by adding 6 µl of the reconstituted 1mM Aromatase Substrate stock solution and 50µl of the reconstituted 10 mM β-NADP+ Stock (100X) to 1444 µl of Aromatase Assay Buffer for a total volume of 1.5 ml.

	Untreated (100% Aromatase activity)	**0% Aromatase activity**	**Test/ Standard sample**
	Group I	**Group II**	**Group III**
Recombinant Human Aromatase	25µl	--	25µl
Letrozole Solution /Test Compounds (10µM,1µM,0.1µM)	--	--	20µl
Aromatase Assay Buffer	25µl	25µl	25µl
Aromatase substrate	30µl	30µl	30µl

Fluorescence based aromatase activity detection was accomplished by utilizing fluorogenic aromatase substrate. The reaction was carried out in total volume of 100µl in 96 well plate. Each reaction was consisting of 25 µl of recombinant aromatase enzyme, 30 µl fluorogenic aromatase substrate, 25 µl Aromatase assay buffer and 20 µl of test sample or Letrozole. Test sample was added at concentration of 10 µM, 1 µM and 0.1 µM in three separate reactions to achieve inhibitory effect at these concentrations. In addition to that one –inhibitor (without Letrozole or test sample) reaction was also set and considered as 100% Aromatase activity. In another reaction only Letrozole was added in order to

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compare the inhibitory effect of test sample with Letrozole. A reaction mix of Aromatase substrate (30µl) and Aromatase assay buffer (70µl) was used for baseline correction by BMG Fluostar Elisa Plate Reader. Reaction mix were incubated at 37°C for 10 min which allowed inhibitor Letrozole or test compounds to react with aromatase enzyme and the fluorescent intensity of each reaction was measured at Ex/Em = 488/527 nm on BMG Fluostar (Germany) on % inhibition mode. IC50 values were calculated by the plotting nonlinear regression graph between percent Inhibition Vs. log concentration by using Graph-Pad Prism software (ver.7).

RESULT AND DISCUSSION:

MOLECULAR DOCKING STUDY

For docking study, the crystal structure of human placental aromatase complexed with breast cancer drug Exemestane was selected for study, as only Exemestane complexed with aromatase enzyme available in protein data bank. The docking score with binding energy and their corresponding intermolecular energy, electrostatic energy, hydrogen bonding and hydrophobic interaction for each class of flavonoids with aromatase enzyme (PDB ID: 3S7S) are given in Table 5.1. Fig.5.1 shows the interaction of Exemestane (steroidal aromatase inhibitor) with aromatase enzyme. The *In silico* predicted active sites for target protein (PDB ID: 3S7S) are MET374, ARG115, LEU372, LEU477, PHE134, VAL370, THR310, PHE221, VAL369, ASH309, SER478, ALA309, ALA306,

TRP224, ILE305, HEM600 and VAL373. The **keto (-C=O) group of Exemestane interacted with MET 374 by H-bonding.**

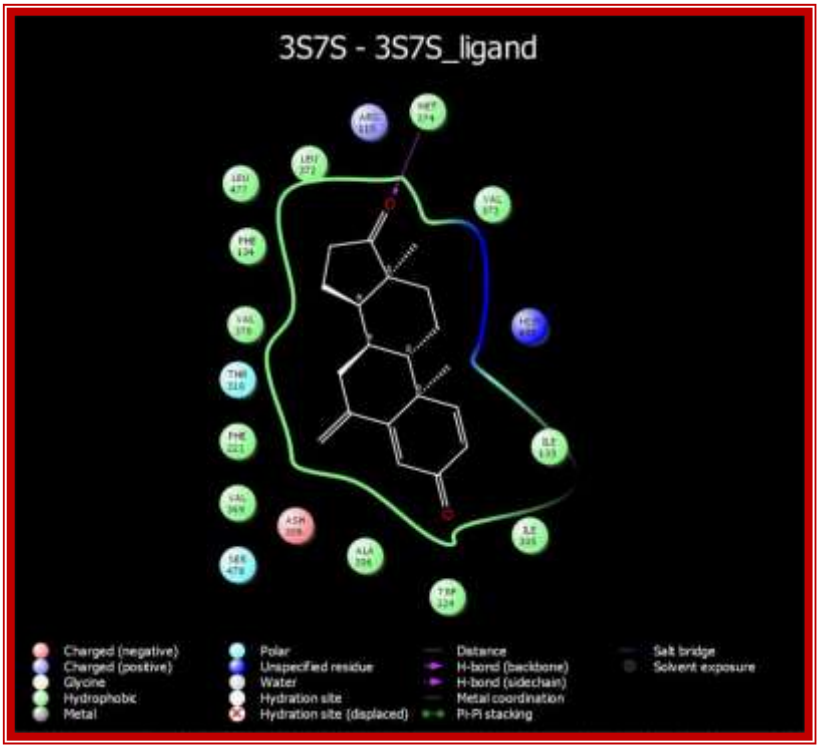

Fig.5.1 Interaction of Exemestane with Aromatase Enzyme

Docking analysis of *2-phenyl-4H-chromen-4-one* derivatives (2A-2S)

The flavones (2A-2S) derivative's binding energies were in the range ~ –8.70 to - 4.839 kcal/mol. (Table 5.1) Among them, flavone (2K) derivative possessed lowest binding energy than selected ligand (Exemestane). Fig.5.2 shows the common binding interactions of 2K with the aromatase enzyme like; pyridine rings "N" atom exhibits the H-bonding interaction with MET 374, aromatic π- π stacking observed with PHE134 and HEM600 (Heme coordinating moiety) and other derivatives like; 2A, 2B, 2E, 2G and 2S were also possessed significantly good binding energy compared to ligand.

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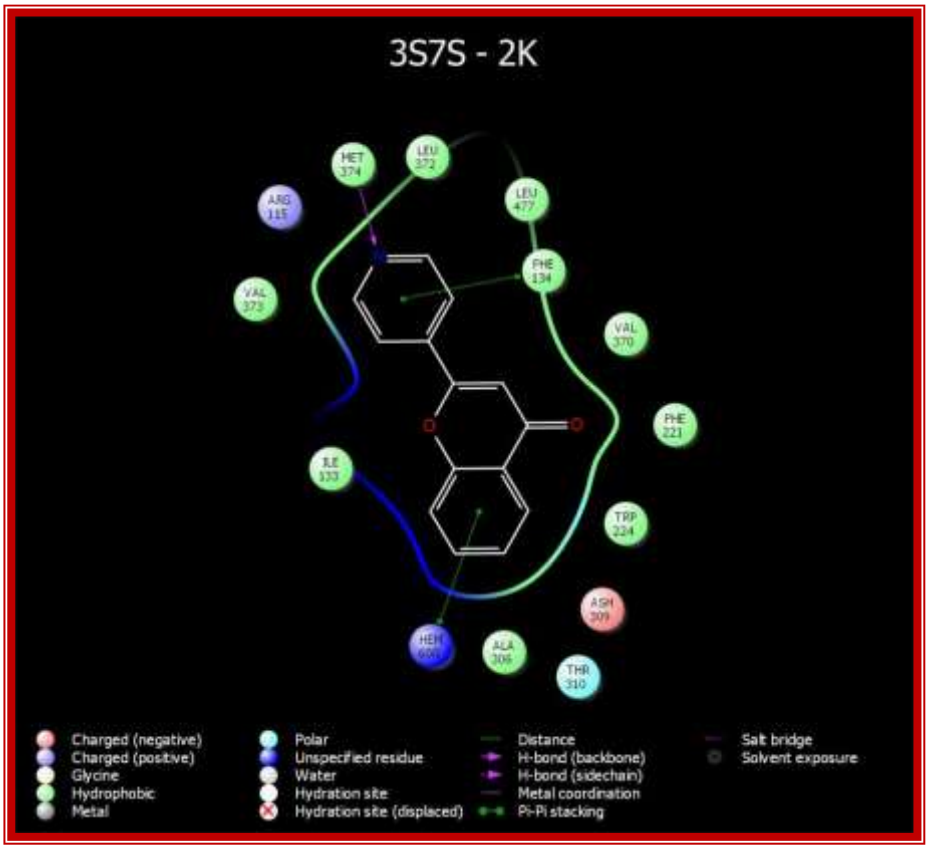

Fig.5.2 Interaction of Flavone derivative 2K with Aromatase Enzyme

Table 5.1 Docking results of *2-phenyl-4H-chromen-4-one* derivatives (2A-2S)

Compound Id	**Docking score**	**Glide energy**	**Glide emodel**	**XP HBond**	**XP PhobEn**	**XP Electro**
2K	-8.70	-31.41	-51.70	-0.70	-0.78	-0.27
2B	-8.00	-37.04	-54.90	-0.68	-1.01	-0.32
2M	-7.90	-35.09	-15.60	0.00	-1.46	0.02
2E	-7.24	-33.72	-44.20	0.00	-0.77	-0.23
2N	-6.92	-34.50	-41.70	-0.70	-0.30	-0.14
2G	-6.69	-35.77	-40.60	-0.04	-0.79	-0.07
2A	-6.68	-34.81	-23.30	0.00	-0.75	-0.14
2S	-6.67	-35.35	-51.00	0.00	-0.004	-0.01
2P	-6.33	-35.79	-29.10	0.00	0.00	-0.16
2R	-6.27	-31.91	-48.90	0.00	-0.40	0.01
2F	-6.15	-38.00	-50.80	0.00	0.00	0.03
2I	-6.06	-36.85	-53.60	0.00	-0.37	0.03
2C	-5.99	-35.41	-52.50	0.00	-0.50	0.03
2D	-5.91	-23.27	-10.40	0.00	0.00	0.03
2Q	-5.62	-24.74	-29.30	0.00	0.00	0.12
2H	-5.45	-35.29	-51.14	0.00	0.00	0.02
2J	-4.99	-35.41	-52.50	0.00	-0.50	0.03
2O	-4.91	-23.27	-10.43	0.00	0.00	0.03
2L	-4.84	-18.76	10.59	-0.70	0.00	-0.27
EXE	-8.49	-45.95	-41.80	-0.70	-0.66	-0.20

Synthesis

Synthetic Pathway for synthesis of (2A – 2S) Compounds

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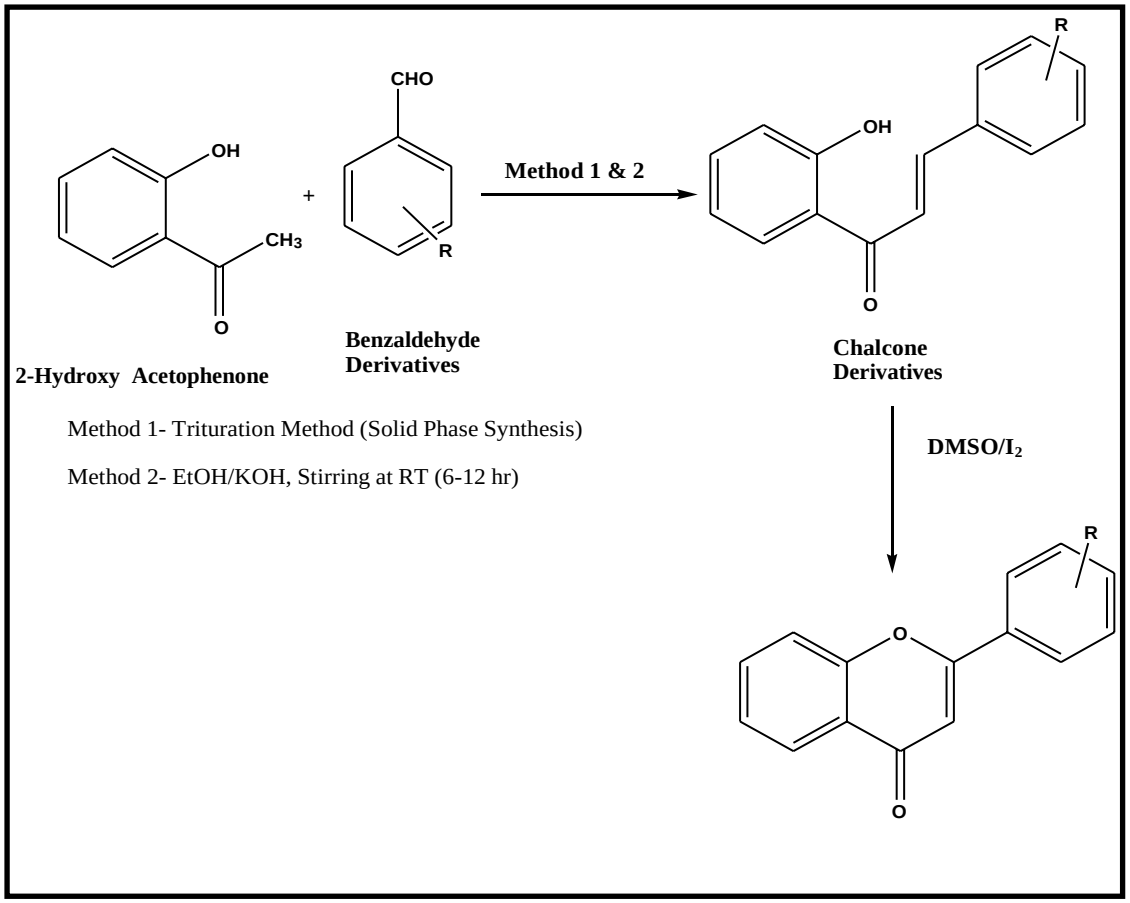

Aldehyde derivatives used for synthesis

A	Verataldehyde	K	4- Pyridine carboxaldehyde
B	3-Hydroxy Benzaldehyde	M	2,4,6 – Trimethoxy Benzaldehyde
C	3-Methoxy Benzaldehyde	N	2,3 – Dimethoxy Benzaldehyde
D	3-Chloro Benzaldehyde	P	2,4 – Dimethoxy Benzaldehyde
E	4- Chloro Benzaldehyde	S	3 – Bromo Benzaldehyde
F	4- Fluoro Benzaldehyde	R	Benzaldehyde
G	4-Methoxy Benzaldehyde		

IR Spectral Characteristics of *(E)-1-(2-hydroxyphenyl)-3-phenylprop-2- en-1-one* derivatives (1A-1G):
The strong (–C=O*str*) peak of all chalcone derivatives observed in the range of 1589 – 1695 $cm^{-1}$and the medium and multiple aromatic (-C-H*str*) and (-C=C*str*) peaks noticed in the range from 3001 – 3088 cm^{-1} and 1465 - 1585 cm^{-1} respectively. The strong and broad (-O-H*str*) peak was noticed between 3200 – 3600 cm^{-1}. The alkyl aryl ether (R-O-Ar) stretching was observed between 1282 - 1159 cm^{-1} in methoxy (-OCH3) substituted chalcone derivatives (1A, 1G).

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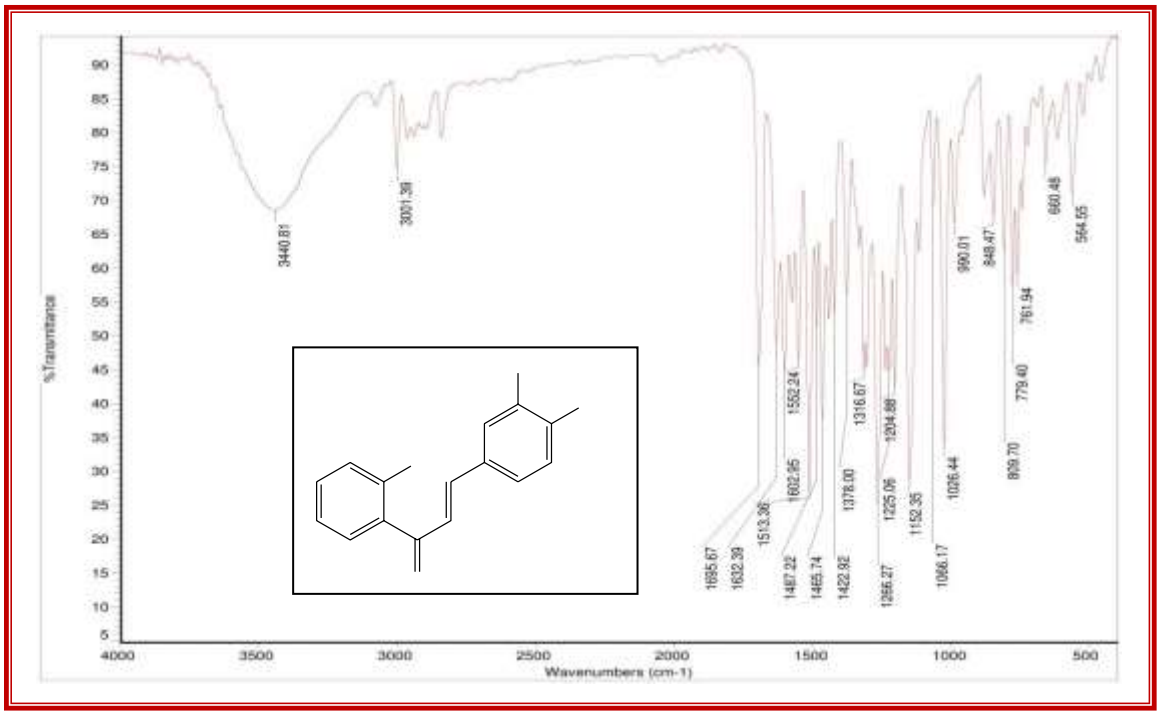

Fig.5.3 IR Spectra of *(E)-3-(3, 4-dimethoxyphenyl)-1-(2-hydroxyphenyl)prop-2- en-1-one* (1A)

Table 5.2 Physicochemical data of *2-phenyl-4H-chromen-4-one* derivatives (2A-2S)

Comp. Id	Molecular Formula	Molecular Weight (g/mol)	Melting Point (^{0}C)	Yield (%)	Rf
2A	C17H14O4	282.09	142 - 145	78	0.56*
2B	C15H10O3	238.24	139 - 142	59	0.38*
2C	C16H12O3	252.08	145 - 148	75	0.45*
2D	C15H9ClO2	256.68	154 - 157	65	0.55*
2E	C15H9FO2	240.23	152 - 154	68	0.46*
2F	C15H9ClO2	256.68	138 - 142	85	0.59*
2G	C16H12O3	252.26	64 - 67	79	0.57*
2K	C14H9NO2	223.23	154 - 157	64	0.44*
2M	C18H16O5	312.32	90 - 94	66	0.39#
2N	C17H14O4	282.29	102 - 105	84	0.52#
2P	C17H14O4	282.29	80 - 84	76	0.46#
2R	C15H10O2	222.24	92 - 95	59	0.65#
2S	C15H9BrO2	301.13	112 - 115	68	0.59#

Chloroform: Methanol (9:1), #Hexane: Ethyl Acetate (8:2)

Spectral Characteristics of *2-phenyl-4H-chromen-4-one* Derivatives (2A- 2S)

The strong (–C=O*str*) peak of every flavone derivative observed in the range 1597 – 1653 cm^{-1}and the medium and multiple aromatic (-C-H*str*) and (-C=C*str*) peaks noticed in the range of 3030 – 3089 cm^{-1} and 1461 - 1586 cm^{-1} respectively. The strong and broad (-O-H*str*) peak of 2B derivative observed between 3200 – 3600 cm^{-1}. The alkyl aryl ether (R-O-Ar) stretching observed in the range 1280 - 1134 cm^{-1} in methoxy (-OCH3) substituted flavonoids (2A, 2G, 2M, 2N and 2P). The mass spectra of all derivatives were observed in the form of molecular ion peak (M^+), M+1 and M+2 peaks. The chlorine substituted compounds (2D and 2F) showed molecular ion peak (M^+) and M + 2 peaks at 57.2 and 259.1, respectively, in tune with the isotopic abundance of 1:3.

The bromine substituted compound 2S showed molecular ion peak (M^+) at 301.2 and M+2 peak at 303.2 due to the isotopic abundance of bromine having the same intensity.. The represented spectra (IR and Mass) is presented in Fig.5.6 – 5.7.

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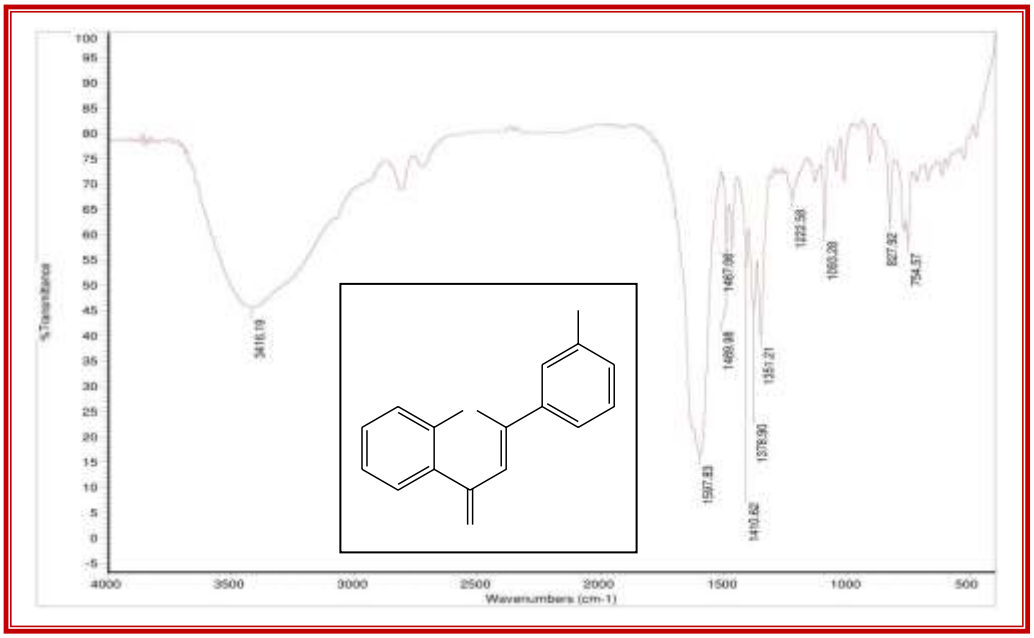

Fig.5.6 IR Spectra of *2-(3-hydroxyphenyl)-4H-chromen-4-one* (2B)

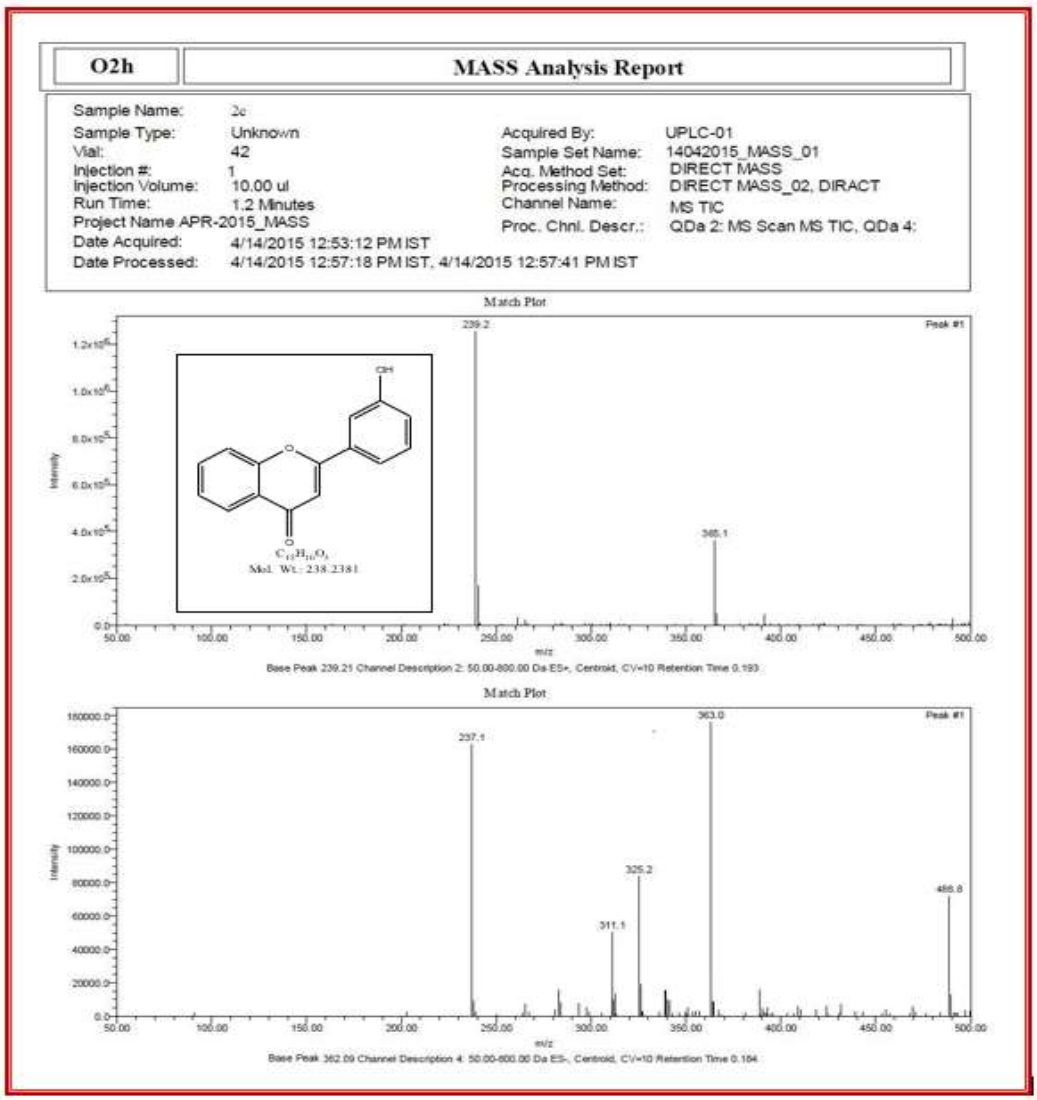

Fig 5.7 show Mass Spectra of *2-(3-hydroxyphenyl)-4H-chromen-4-one* (2B)

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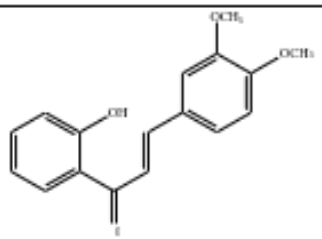
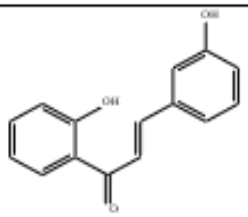
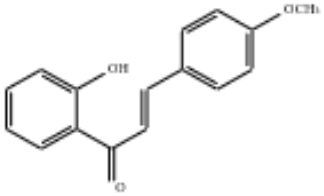
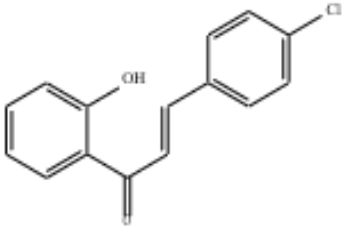
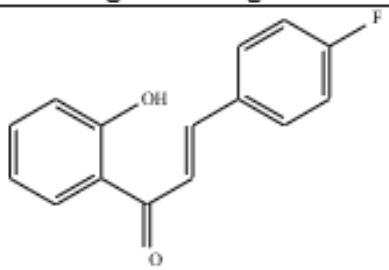
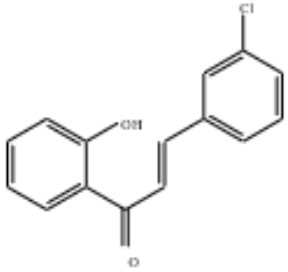
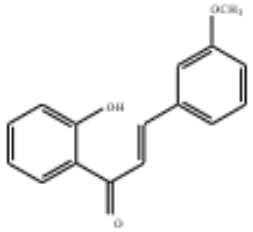
Table 5.3 Physicochemical and IR spectral data of *(E)-1-(2-hydroxyphenyl)-3- phenylprop-2-en-1-one derivatives* (1A-1S

Id	Structural Characteristic	M.P (^{0}C)	Yield (%)	*R_f	IR (cm^{-1})
1A	 (E)-3-(3,4-dimethoxyphenyl)-1-(2-hydroxyphenyl) prop-2- en-1-one Mol. Formula: $C_{17}H_{16}O_4$ Mol. Weight: 284.31 g/mol	92 - 94	86	0.45	3001 (Aromatic C-H*str*), 3600-3200 (O-H*str*), 1695 (-C=O*str*) 1513,146 5(-C=C*str*), 1266,1152 (R-O-Ar *str*)
1B	 (E)-1-(2-hydroxyphenyl)-3-(3-hydroxyphenyl) prop-2-en-1-one Mol. Formula: $C_{15}H_{12}O_3$ Mol. Weight: 240.25 g/mol	85 - 88	78	0.39	3030 (Aromatic C-H*str*), 3600-3200 (O-H*str*), 1640 (-C=O*str*), 1565, 1488 (C=C*str*)
1C	 (E)-1-(2-hydroxyphenyl)-3-(4-methoxyphenyl) prop-2-en-1-one Mol. Formula: $C_{16}H_{14}O_3$ Mol. Weight: 254.28 g/mol	78 - 80	83	0.56	3065 (Aromatic C-H*str*), 3600-3200 (O-H*str*), 1637 (-C=O*str*), 1581,1488 (C=C*str*) 1261,1152 (R-O-Ar *str*)
1D		82 - 84	68	0.48	3055 (Aromatic C-H*str*), 3600-3200 (O-H*str*), 1635 (-C=O*str*),

	(E)-3-(4-chlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one Mol. Formula: $C_{15}H_{11}ClO_2$ Mol. Weight: 258.7 g/mol				1558,1488 (C=C*str*) 746 (Ar-Cl*str*)
1E	 (E)-3-(4-fluorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one Mol. Formula: $C_{15}H_{11}FO_2$ Mol. Weight: 242.25g/mol	68 - 70	85	0.38	3055 (Aromatic C-H*str*), 3600-3200 (O-H*str*), 1589 (-C=O*str*), 1513,1494 (C=C*str*)
1F	 (E)-3-(3-chlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one Mol. Formula: $C_{15}H_{11}ClO_2$ Mol. Weight: 258.7 g/mol	80 - 83	75	0.59	3056 (Aromatic C-H*str*), 3600-3200 (O-H*str*), 1647 (-C=O*str*), 1583,1491 (C=C*str*) 755 (Ar-Cl*str*)
1G	 (E)-1-(2-hydroxyphenyl)-3-(3-methoxyphenyl) prop-2-en-1-one Mol. Formula: $C_{16}H_{14}O_3$ Mol. Weight: 254.28 g/mol	65 - 68	88	0.61	3010 (Aromatic C-H*str*), 3600-3200 (O-H*str*), 1640(-C=O*str*), 1576,1486 (C=C*str*) 1271,1157 (R-O-Ar *str*)

***Mobile Phase:** Hexane: Ethyl Acetate (7:3)

Physical Characteristics of *2-phenyl-4H-chromen-4-one* Derivatives (2A- 2S)
Melting points of synthesized flavonoids were ranged from 64 -157^{0}C and the

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%yield of them were ranged from 65% to 92%. All these synthesized compounds were soluble in methanol, ethanol and DMSO. (Table 5.4)

Table 5.4 Physicochemical data of *2-phenyl-4H-chromen-4-one* derivatives (2A-2S)

Comp. Id	Molecular Formula	Molecular Weight (g/mol)	Melting Point (^{0}C)	Yield (%)	Rf
2A	C17H14O4	282.09	142 - 145	78	0.56*
2B	C15H10O3	238.24	139 - 142	59	0.38*
2C	C16H12O3	252.08	145 - 148	75	0.45*
2D	C15H9ClO2	256.68	154 - 157	65	0.55*
2E	C15H9FO2	240.23	152 - 154	68	0.46*
2F	C15H9ClO2	256.68	138 - 142	85	0.59*
2G	C16H12O3	252.26	64 - 67	79	0.57*
2K	C14H9NO2	223.23	154 - 157	64	0.44*
2M	C18H16O5	312.32	90 - 94	66	0.39#
2N	C17H14O4	282.29	102 - 105	84	0.52#
2P	C17H14O4	282.29	80 - 84	76	0.46#
2R	C15H10O2	222.24	92 - 95	59	0.65#
2S	C15H9BrO2	301.13	112 - 115	68	0.59#

Chloroform: Methanol (9:1), #Hexane: Ethyl Acetate (8:2)

SUMMARY AND CONCLUSION

Cancer is a major public health concern worldwide and is the second leading cause of death after cardio vascular diseases. Globally, nearly 1 in 6 deaths occur due to cancer. By 2050, the global burden is expected to grow to 27 million new cancer cases and 17.5 million cancer deaths simply due to the growth and aging of the population. As per WHO and GLOBOCAN reports of more and less developed regions of the world, breast cancer is the most common type of cancer in women among all other types of cancer. As per PBCR (Population Based Cancer Registry) data, the breast cancer accounts for 25% to 32% of all female cancers across India. In women, breast cancer is the second cause of cancer death after lung cancer, the world over.

From last three decades, scientists have been studying natural and synthetic Flavonoids and their derivatives for their breast cancer activity by inhibiting the aromatase enzyme. Many papers have reported the activity of Flavonoid derivatives against breast cancer in articles, patents and other authentic online sources. The Structure-Activity Relationship of Flavonoids for their breast cancer activity was derived from work of literature. 76 Flavonoid derivatives were designed based on available literature and SAR database of Flavonoids. The novelty of designed flavonoids was checked by Sci-Finder and Pub Chem database. The designed Flavonoids were further screened by Molecular Docking by Glide software, Maestro Suit 10.4, Schrodinger. 40 compounds were further selected on the basis of docking score against selected ligand (Exemestane) for the synthesis purpose and their *in- silico* ADMET properties were checked.

The first step in the synthesis of Flavonoids was synthesis of chalcone derivatives by Aldol Condensation. Chalcone (1A –1S) were synthesized by trituration and KOH/EtOH method using KOH as base and EtOH as solvent. Other chalcones (3B-3S), (5B – 5S) and (7B-7S) were synthesized by Ba(OH)2,

Li(OH)2 and 60% KOH in Ethanol as the base. The second step was ring cyclization by catalytic amount of iodine in DMSO as a solvent.

The physicochemical parameters of all synthesized compounds were determined. The % yield of Flavonoid derivatives were ranged from 49 - 94% and melting point of synthesized flavonoids were ranged from 64 - 207^{0}C. The sharp melting point indicative of purity of compounds. The structure of Flavonoids were confirmed by IR and Mass spectroscopic techniques.

Flavonoids are reported to exhibit anti-oxidant activity. Hence, further proving the same, *In vitro* Antioxidant activity was carried out by DPPH assay. 30 compounds were tested for their anti-oxidant activity and their IC50 value was calculated by plotting a graph of % Inhibition Vs. Concentration.

18 compounds with higher anti-oxidant activity were tested for their cytotoxicity study on breast cancer cell line (MCF-7) by MTT assay using Letrozole the standard. The IC50 values were calculated by plotted nonlinear graph between % cell inhibition Vs. log concentration using Graph Pad Prism software. The IC50 value of Letrozole was found to be 30.39 µM, while IC50 value of 6B, 2K, 4K, 6K, 4B, 2B and 4C were found to be 0.35 µM, 1.64 µM, 15.75 µM, 16.08 µM,

16.08 µM, 20.73 µM and 22.02 µM respectively. These flavonoids were considered as more potent than Letrozole.

Based on docking score and cytotoxicity data by MTT assay, 06 compounds namely 2B, 2K, 6B, 6K, 4B and 4K were selected for *In-vitro* Aromatase inhibitory activity by Fluorogenic Assay Kit. The % inhibition of test compounds and standard drug (Letrozole) were measured at three different concentration 0.1, 1.0 and 10.0 µM. Based on % inhibition data, the IC50 value of standard and test compounds were calculated by the plotted nonlinear graph between %

inhibition Vs. log concentration by using graph pad prism software. The IC50 value of Letrozole was found to be 0.86 µM, while IC50 values of compounds 2B and 6B were found to be 0.31 µM and 0.36 µM. Hence, it was concluded that, compounds 2B [2-(3-hydroxyphenyl)-4H-chromen-4-one] and 6B [2-(3- hydroxyphenyl)-4H-benzo[h]chromen-4-one] exhibited potent aromatase inhibitory activity than Letrozole as marketed aromatase inhibitor drug. While the other compounds 2K, 4B, 6K and 4K showed comparable activity with respect to Letrozole based on their IC50 values of 2K (0.93 µM), 4B (0.98 µM) 1061 µM (6K) and 3.06 µM (4K).

CONCLUSION

In conclusion, a series of 40 flavonoid derivatives were synthesized from 76 designed compounds by docking. The *in silico* ADMET properties of flavonoids were predicted by QikProp software and found all compounds having drug likeness properties. The novelty of all compounds was checked by Sci-Finder and Pub Chem database. Chalcone synthesis were carried out using different base like; KOH, Ba (OH)2 and Li (OH)2 by aldol condensation reaction and flavonoids were synthesized by addition of iodine with DMSO as solvent. 18 compounds were evaluated for their *in vitro* cytotoxicity study and their IC50 value compared with standard drug Letrozole as marketed aromatase inhibitor. The IC50 value of Letrozole was found to be 30.39 µM, while IC50 values of other synthesized flavonoids 6B, 2K, 4K, 6K, 4B, 2B and 4C were found to be 0.35 µM, 1.64 µM, 15.75 µM, 16.08 µM, 16.08 µM, 20.73 µM and 22.02 µM respectively. Hence, 07 flavonoids out of 18 were considered as more potent than Letrozole. 06 compounds 2B, 2K, 6B, 6K, 4B and 4K were selected based on cytotoxicity and docking result and further tested for In-*vitro* Aromatase inhibitory activity by fluorogenic assay kit. The IC50 value of compounds 2B [2-(3-hydroxyphenyl)-4H- chromen-4-one] and 6B [2-(3-hydroxyphenyl)-4H-benzo[h]chromen-4-one] were found to be 0.31 µM and 0.36 µM. Hence, it was concluded that, compounds 2B and 6B exhibited potent aromatase inhibitory activity compared to Letrozole (IC50 = 0.86 µM). While the other compounds 2K, 4B, 6K and 4K were considered to comparable inhibitory activity with respect to Letrozole. The common structural features present in 2B, 2K, 6B, 6K, 4B and 4K flavonoids are 3- hydroxyl Benzaldehyde (B Series) and 4 - Pyridine carboxaldehyde (K Series) derivatives. The resulted flavonoids mimic the steroidal substrate, though it is non-steroidal structure. Hence, these potent flavonoids might be promoted to reduce serious side effect caused by steroidal aromatase inhibitors. Their considerable aromatase inhibitory activities make them a good candidate for the development of aromatase inhibitors.

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