**Cellular and *in- silico* studies implicate anti-breast cancer activity of *Erythrina variegata* extracts in MCF7- cell lines.**

**Abstract**

According to epidemiological research, breast cancer is the fifth leading cause of cancer-related deaths worldwide and is more frequently diagnosed than lung cancer. There is growing awareness of the adverse effects of radiation and immunotherapy on several organs, as well as the variety of symptoms that can recur. Several natural compounds are being employed as adjuncts or in therapy as part of an alternative treatment regimen that is currently being investigated worldwide. Additionally, a number of compunds are undergoing various phases of clinical testing. Using cellular and *in silico* experiments, the current work investigates the possibility of *Erythrina variegate* extracts eliciting anti-breast cancer action. Alkaloids and flavonoids are the plant's main ingredients, according to phytochemical research. Based on our data, the cytotoxicity is effective, with IC50 values of 85.27 ug/ml/24 hours after incubation. Additionally, by lowering the percentage of cells at the G0/G1 phase, the extracts caused necrosis in 3.6% of cancer cells and death in 49.37% of them. The cell-cycle assay showed greater inhibition at the S and G2/M phases. Lastly, quantitative PCR demonstrated a considerable inhibition of the expression of the apoptotic marker genes *P53* and *Caspase 3*. The interactions involving ligands viz. HER2/Osajin, eEF-2K/alpinumisoflavone, and PDF/wighteone had the best docking scores, with -8.9, -8.7, and -8.4 respectively. Finding the tree's precise chemical components and cellular mechanisms will be made possible by additional thorough phytochemical characterization, cellular assays, and simulations. Cumulatively, the initial findings support the compounds' anti-breast cancer properties and their potential as a substitute treatment for breast cancer.

**Key words-** *Erythrina variegate*, Estrogen or Progesterone receptor positive, human epidermal growth factor receptor 2 (HER2), Wnt signaling, MCF7-Human breast adenocarcinoma cell lines.

**Introduction**

An illness known as cancer occurs when body own cells proliferate out of control and spread to other tissues and organs. In addition to invading adjacent tissues, cancerous tumors have the ability to metastasize, or spread, to other parts of the body to generate new tumors (Arun Upadhyay 2020). Breast cancer occurs when cells proliferate uncontrollably. Depending on which breast cells develop into cancer, there are various types of breast cancer. The ducts or lobules are where the majority of breast cancers start. According to epidemiological research, with an estimated 2.3 million cases and 685,000 deaths worldwide, breast cancer has surpassed lung cancer as the most often diagnosed cancer and the fifth leading cause of cancer-related fatalities and it is anticipated that by 2070, there would be 4.4 million instances (Zohre Momenimovahed and Hamid Salehiniya 2019). Breast cancer ranked first for incidence and mortality among women in most nations in the world in 2020, accounting for about 24.5% of all cancer cases and 15.5% of cancer deaths (Evelina Arzanova and Harvey N. Mayrovitz 2022). Gender, age, reproductive factors, age at menarche and menopause, pregnancy, abortion, ovulatory menstrual cycle, postmenopausal hormone therapy, genetic factors, family history, and various environmental factors, such as smoking and alcohol use, are the main risk factors for breast cancer (Sergiusz Łukasiewicz et al., 2021; Leila Allahqoli et al., 2022). Endocrine receptor (either estrogen or progesterone receptor) positive, human epidermal growth factor receptor 2 (HER2) positive, triple positive (either estrogen, progesterone, or HER2 receptor positive), and triple negative (no estrogen, progesterone, or HER2 receptors) are the four main categories of breast cancers based on immune-histochemical classification of hormone receptor status in the cancerous breast cells (Stuart J Schnitt 2010). According to the expression levels of endocrine receptors, proliferative genes, and oncogenes, global gene expression studies further divide people into different molecular classes: luminal A (ER+/PR+ and Ki67 high), luminal B (ER+/PR+, Ki67 low or, ER+/PR+/HE R2+), HER2+, basal (ER-/PR- /basal myoepithelial markers high/EGFR+), and normal breast-like (ER-/PR-/basal myoepithelial markers-/EGFR-) (Mahmoud Al-Balas et al., 2024). Germline mutations in breast cancer (BCs) susceptibility genes, such as BRCA1, TP53, PTEN, and several other genes, are the primary cause of acquiring hereditary breast cancer. The two types of BC susceptibility genes—high- and low-penetrance genes—interact with many genes and environmental variables (Mahdavi et al., 2018).

According to Gegechkori et al. (2017), lymphedema, cardiotoxicity, exhaustion, neuropathy, cognitive impairment, endocrine disturbances, sexual health problems, and mental health problems are typical cancer side effects. Immune-related side-effects that impact various organs, such as the skin (rash, pruritus) or gastrointestinal system (diarrhea, colitis), are the primary disadvantage of immunotherapies (Marilina García-Arandaa and Maximino Redondo 2019).

Furthermore, side symptoms such vaginal dryness, hot flashes, non-alcoholic fatty liver disease, and an elevated risk of endometrial cancer are seen by survivors undergoing hormone therapy (Boing et al., 2020). Last but not least, radiation therapy, which employs high-energy rays to destroy cancer cells, only affects cells in the body that receives the radiation treatment. This raises concerns about resistant cells in the breast or armpit area following surgery. Additional adverse effects include skin irritation similar to a sunburn, heaviness in the breasts, discoloration, redness, or a bruised appearance, and overall exhaustion (Sowunmi, Anthonia C et al., 2020). These drawbacks emphasize the need for safe and efficient alternative therapy approaches.

Natural coumpunds with strong anticancer properties and minimal harm to healthy cells have become attractive options for cancer treatment in recent years (Pritee Chunarkar-Patil et al., 2024). They are also being extensively investigated for drug discovery and development due to their advantageous properties to overcome a number of synthetic drug limitations, including side effects, bioavailability, targeted administration, and dose (Sohel M, Biswas PS et al., 2022).

The Wnt signaling route is one of the many cell signaling conserved signaling pathways which are essential for regulating the development of embryos and organs. Additionally, they are also implicated to the advancement of cancer. The wnt proteins are lipo-glycoproteins that play essential roles in regulating tissue patterning, stem cell self-renewal, cell-cell interactions, and cell specification throughout embryonic development (Jiaqi Liu et. al., 2022). Distinct downstream pathways are activated by Wnt proteins (Wnts) coupling to distinct receptors (Nusse R and Clevers H 2017). According to recent research, Wnt signaling plays a critical role in the regulation of the immune milieu, stemness maintenance, treatment resistance, and phenotypic formation of breast cancer. The three factors—Wnt/β-Catenin, Wnt–planar cell polarity (PCP), and Wnt Ca2+ signaling—have distinct roles in the development of breast cancer and share overlapping components (Xiufang et al., 2020). A key player in carcinogenesis and development, the PI3K/AKT/mTOR signaling pathway is constitutively active in a number of cancer processes. Second, cancer cell survival, proliferation, migration, and response to therapy are all regulated by the PI3K/AKT/mTOR pathway (Alzahrani et al., 2019). The signaling pathway involving phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), and mammalian target of rapamycin (mTOR) is implicated in apoptosis, invasion, migration, glucose metabolism, DNA repair, and cell proliferation (Miricescu et al., 2021). Natural products can alter Wnt signaling and present prospective therapeutic options, according to a number of studies (Dan Liu et al., 2019; Yu WK 2020).

There are roughly 110 species of trees and shrubs in the genus Erythrina that are generally known as "coral trees" (Manuel Duenas-Lopez 2022). Originating in Indonesia and Malaysia, coral trees are native to the Old World tropics and have now expanded to eastern Polynesia (the Marquesas). Variegata, the most appealing variety, is grown for both its seasonal, eye-catching red blossoms and its variegated leaves. Research on the phytochemicals of *Erythrina variegata* species has shown that alkaloids and flavonoids are important components. For example, *Erythrina indica* has been found to include alkaloids and aliphatic fatty acids (Aimé G. Fankam and Victor Kuete 2024). Traditional medicine has utilized various portions of *E. variegata* as nervine sedatives, febrifuges, anti-epileptics, and anti-asthmatics. Additionally, it may be used to treat conditions including helminthiasis, bacterial infections, inflammation, sleeplessness, coughing, cuts, and wounds (Preeti Kumari, et al., 2017).
Numerous researchers suggest that the herb has anticancer properties. According to John et al. (2021), methanol extract has anticancer potential against human breast cancer cell lines via downregulating Bcl-2 expression and using an intrinsic apoptotic mechanism. Next, Vaishali Rai M et al. (2017) show antioxidant activity against the human colon cancer cell line (HT-29) by DPPH, superoxide anion scavenging, metal chelator, and nitric oxide scavenging activities. Lastly, anticancer activity against the adenocarcinomic human alveolar basal epithelial cancer cells (A549) is reported by Priya et al. (2018).

A structure-based approach to drug creation includes molecular docking models, molecular interactions and forecasts the mechanism and affinity of receptor-ligand binding. The field of drug design research has made extensive use of this technology in recent years (Fan et al., 2019). Predicting the energy profile (like binding free energy), strength, and stability (like binding affinity and binding constant) of complexes can be done using information gleaned from the preferred orientation of bound molecules. The molecular docking scoring function can be used for this. In order to reduce the free energy of the entire system, the primary goal of molecular docking is to obtain an optimal docked conformer of both interacting molecules (Agarwal and Mehrotra 2016). Carcinoembryonic antigen (CD66d), eukaryotic elongation factor 2 kinase (eEF2K), estrogen receptor α (ERα), human mitochondrial peptide deformylase (PDF), and HER2 (human epidermal growth factor receptor 2) were all chosen as potential cancer ligands for this investigation.

The study's primary goal is to assess the phytoconstituent's anti-cancer potential *in vitro* using cell lines and correlate the findings using *in-silico* simulations using ligands from different databases.

**Materials and methods.**
**Collection, preparing, and extracting plant material.**
The plant's leaves were gathered in rural Bengaluru (Latitude-12.971599,Longitude-77.594566, DMS Lat-12° 58' 17.7564''N, DMS Long-77° 35' 40.4376'' E. After taxonomist verification of specimen, the leaves were allowed to dry in the shade for five to six days. The dried materials were ground into a powder using a household blender and then sieved through laboratory-grade sieve number 45. The extraction was carried out in a Soxhlet extractor with methanol as the solvent. After being concentrated, the extract was examined further.

**In-vitro studies**

Cell lines culture-MCF7-Human breast adenocarcinoma cell lines were procured from (NCCS, Pune), and cultured in 96 well culture plates with DMEM- High Glucose Cell culture medium with 10% FBS along with the 1% antibiotic antimycotic solution in the atmosphere of 5% CO2, 18-20% O2 at 370C temperature in the CO2 incubator and sub-cultured for every 2-3days.

MTT Assay- MTT colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow-coloured water-soluble tetrazolium dye MTT to formazan crystals. Care was taken to avoid experimental and human errors. % Cell viability was calculated using the formula: % cell viability = [Mean abs of treated cells/Mean abs of Untreated cells] x 100 11.

The IC50 value was determined by using linear regression equation i.e., Y =Mx+C. Here, Y = 50, M and C values were derived from the viability graph.

In this study, 1 test compound was evaluated to analyse the cytotoxicity effect on MCF7 cells. The concentrations of the test compound used to treat the cells are as described in table-1

Apoptosis assay - FITC Annexin V is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. Propidium Iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells.

Cell Cycle study - The most widely used dye is propidium iodide (PI), which has red fluorescence and can be excited at 488 nm. Propidium iodide (or PI) is a fluorescent intercalating agent that can be used to stain cells. It is used as stain in flow cytometry to evaluate cell viability or DNA content in cell cycle analysis.

Gene expression study- RNA concentration in different culture conditions was quantified by Nano drop. The Primers designed for the respective gene expression studies were as mentioned in the table-2. All the primers (HPLC grade) were synthesized based on available literature and procured from our outsourcing partner Eurofins, Bangalore. Gene expression study was carried out by relative quantification using Real Time PCR: The relative quantification of the gene expression was done in QuantStudio3 system (Thermo Fisher) using the SYBR Green Chemistry (Sensifast SYBR HiRoxkit, Bioline, USA) with endogenous control gene (beta-actin) using ∆Ct method. ∆Ct = Average Ct of test sample - Average Ct of calibrator The ∆Ct values will be converted to a linear form using the formula:

E-∆Ct, Where E=amplification efficiency.

To calculate the expression of a target gene (TG) relative to the EC, the comparative Ct (∆∆Ct) method (Step one Software v2.2.2) was used following the equation:

∆∆Ct = (Ct Target gene)-(Ct EC) – (Ct Target gene)-(Ct EC). Test Sample Calibrator The ∆∆Ct values were converted to a linear form using the formula:

E –∆∆Ct Relative expression is a variation of the expression of a gene between two samples. The RQ (Relative quantification value) is the fold change compared to the calibrator (untreated sample, time zero, etc.).

***In silico* analysis**

Lipinski's rule of five is frequently applied in drug discovery since it predicts the chemical and physical characteristics of bioavailability and allows for the prediction of biologically active molecules. In pharmacokinetic drug properties, the Lipinski rule is based on certain physicochemical properties, such as molecular mass <500 Da, high lipophilicity (Log<5), fewer than five hydrogen donors, fewer than ten hydrogen bond acceptors, and molecular refractivity 40-130. To be employed, the compounds must meet at least three of the aforementioned requirements (Lipinski et al., 2001). To determine if the phytoconstituents adhere to Lipinski's rule, they were analyzed. Preparation of ligand - the structures of phytoconstituents of the leaves of *Erythrina indica* were downloaded from Indian Medicinal Plants Phytochemistry and its Therapeutics (IMPPAT) database (https://cb.imsc.res.in/imppat/ assessed on March 2024) in PDB format. For target preparation the breast cancer target structure was downloaded from Therapeutic Target Database (TTD) ( https://idrblab.net/ttd/ assessed on March 2024) in PDB format. The water molecules were removed from the target molecule and polar hydrogen were added later, Kollman charges were added to the target protein. The active sites for the target proteins were found using Qsite software. 4.1.2.4(ref).
Docking analysis -Docking studies were performed using the AutoDock Vina tools(J. Eberhardt et al., 2021). Various docked confirmations were obtained. From the docked structures, best ligand-receptor assemblies were selected based on lowest energy and the docking scores were then tabulated.

**RESULTS**

**In vitro studies**

MTT Assay

% cell viability values of Methanolic extract treated on MCF7 cells after the treatment period of 24hrs are described. The results of cytotoxicity study performed by MTT assay suggest that the test compounds, viz., Methanolic extract was effectively cytotoxic in nature with IC50 values of 85.27ug/ml on Human breast cancer (MCF7) cells after the incubation period of 24 hours (Figure-1, table-3).

Apoptosis assay observations suggested us that the test compound, Methanolic extract was significantly enhanced the 49.37% of apoptosis and 3.6% necrosis in human breast cancer cells (Figure-2,3 table-4). Doxorubicin showed similar trend of apoptotic potential against human breast cancer cells with high % apoptosis and necrosis and further studies like ROS study and Cell cycle study need to be performed to confirm the mechanism of action behind the apoptotic effect of these compounds on Human breast cancer cells *in vitro*.

Cell cycle analysis

Methanolic extract showed significant cell inhibition after the treatment period of 24hrs, we have selected IC50 concentration against the MCF7 cell lines; we evaluated Cell Cycle study by Flow Cytometry to check the phases of cell cycle arrest and the obtained results by Flow Cytometry .The MCF7 cells treated with Methanolic extract with IC50 concentration showed increased % cells at S and G2/M phases by inhibiting the % cells at G0/G1 phase as compared to control group (figure-4, table-5). It could be concluded that the Methanolic extract will not allow the cells to undergo cell division or proliferate in Sub G0/G1 and G2/M phase arrest and may be considered as a potent anti-breast cancer drug. Further, the effective cell cycle arrest at Sub G0/G1 and G2/M phase was similar to the anti-cancer drug Doxorubicin.

Gene expression study

To accurately and reliably determine the gene expression values raw fluorescence data (Ct values) generated by the real-time PCR instrument (Quant Studio3) were exported to Quant Studio3 software to scale raw data to an endogenous control gene (beta-actin). The calibrator had a RQ value of 1 values exceed 1 the gene was considered up regulated otherwise down regulated. Relative mRNA expression of P53, Caspase 3, c-Myb genes in MCF7 cells in different culture groups viz., Untreated, Doxorubicin with 1uM and Methanolic extract with 85ug/ml is depicted in figure-5. The relative gene expression level of cMyb gene(0.07) was down regulated in treated groups compared to untreated group and whereas genes upregulated for Caspase 3(3.31) and p53 genes(4.86) in treated group in comparison to untreated group.

***In silico* studies**

Structure of 25 chemical phytoconstituents of Erythrina indica were downloaded IMPPAT) database in PDB format, also the respective breast cancer target structures TTDin PDB format.

Docking studies

The results of docking the selected phytoconstituents and the ligands are tabulated in table-6. Docking results showed satisfactorily results with all the ligands(Figure-6-9). However, notable and high-binding was found in ligands eEF-2K/alpinumisoflavone(-8.7), ERα/ alpinumisoflavone (-8.6), HER2/Osajin(-6.8), HER2/ CD66d(-8.9) and PDF/wighteone(-8.4) respectively.

**Discussion**

The purpose of the study was to evaluate the anti-cancer potential of *Erythrina indica* leaf extract in methanol. According to the results of the MTT assay, the methanolic extract had an IC50 value of 85.27 ug/ml and was cytotoxic and anti-cancer in nature on human MCF7 breast cancer cells. Subsequently, the results of the apoptosis investigation indicated that the test drug considerably increased the human breast cancer cells' 3.6% necrosis and 49.37% apoptosis. From the cell cycle analysis, it’s could be concluded that the Methanolic extract arrests cell division or proliferate in sub G0/G1 and G2/M phase arrest and may be considered as a potent anti-breast cancer drug similar to the anti-cancer drug Doxorubicin. Using the measured Ct values for each gene in relation to the treated and untreated samples, the fold expression was computed. The results obtained indicate that the expression level of other genes and the relative gene expression level of *cMyb* genes were downregulated in the treated groups when compared to the untreated group. Compared to the untreated group, the treated group's *caspase 3* and *p53* genes were markedly elevated. In conclusion, the real-time quantitative PCR revealed that the expression of the apoptotic markers *Caspase 3* and *P53* was considerably lower than that of the control group.
The ability of cancer cells to evade apoptosis, or programmed cell death, is one of their unique traits, referred to as "cancer hallmarks." The process is typically aided by two signaling pathways, the intrinsic and extrinsic cascades, which are made up of multiple components. If these cascades are altered, the cell may develop a phenotype that is resistant to apoptosis (Sadegh Rajabi et al., 2021). Targeting many molecules in apoptotic pathways can therefore be an effective strategy for both minimizing therapy resistance and discovering new anticancer treatments. While caspase-3 has a well-established role as an executor during apoptotic cell death, p53 (or p53) protein performs the role of a tumor suppressor, regulating cell division by preventing cells from multiplying. Natural compunds have been shown in numerous studies to trigger p53-induced cell cycle arrest and apoptosis, as well as mitochondrial alterations exacerbated by activating the caspase cascade apoptosis in breast cancer cells(Jing Zhang et al., 2024). The transcription factor c-Myb protein controls apoptosis, differentiation, and cell division. It plays important roles in hematopoiesis and aids in the development of cancer. Natural compunds have been shown in studies to activate key cell signaling pathways, such as the WNT and downstream targets (Pablo Angulo et al., 2017). Numerous apoptosis marker genes exhibit gene expression effects due to natural compounds, as demonstrated by expression studies, suggesting that these compounds play a role in modulating apoptotic pathways. In summary, the initial *in vitro* findings implicate the potentialof *Erythrina indica's* as a future compound for additional research on breast cancer.

The chemicals/protein markers that cancer cells produce in greater quantities than healthy cells are known as tumor markers. Theese indicators function as biomarkers that can be used to evaluate the progression or response to treatment, or ascertain cancer. Prognosis evaluation, post-operative surveillance, medication response prediction, and therapy monitoring can all be aided by these markers. The binding affinities of the five most prevalent markers to *Erthrenia variegata* phyto-compounds were examined in this work. A well-known tumor marker for a variety of cancers, including breast cancer, is the glycosylated protein known as the carcinoembryonic antigen (CD66d) (Lisy et al., 2008). In human malignancies, dysregulated overexpression of CD66d is linked to an invasive phenotype and anoikis resistance, which are mediated by excessive TGFβ, AKT, FAK, and SRC signaling (Johnson et al., 2015). The atypical member of the α-kinase family, Eukaryotic Elongation Factor 2 Kinase (eEF2K), is a crucial regulator of multiple cellular processes linked to carcinogenesis. According to studies, it is upregulated in a number of tumors, is linked to poor patient outcomes, and is a target for anticancer drugs (Temme et al., 2021). Induction of eEF2 phosphorylation via mTOR and AMP regulation is closely associated with anti-tumor action (Ballard DJ et al., 2021). It has been established that the most significant target in breast cancer is the estrogen receptor α (ERα). Tamoxifen, a selective ER modulator (SERM), has been a popular therapy and preventative medication for the past 30 years (Ang Luo and Xuan Zhang et al., 2016). One new cancer treatment target that has been suggested is human mitochondrial peptide deformylase (PDF). According to Harsharan Randhawa et al. (2013), PDF is up-regulated in a number of cancer forms, including breast cancer. HER2 (human epidermal growth factor receptor 2) is a transmembrane receptor that belongs to the HER family of receptor tyrosine kinases, which also includes HER1, HER3, and HER4 Yoo-Na Kim et al. (2024). While HER2-transfected cells with wild-type p53 undergo apoptosis soon after transfection and exhibit reduced proliferation after apoptosis-resistant cells stabilize, HER2 overexpression is linked to proliferation when induced in tumor cells with mutant p53 (Casalini et al., 2001).

Docking results showed satisfactorily results with the following combinations of compound-ligand conjugates eEF-2K/alpinumisoflavone(-8.7), ERα/ alpinumisoflavone (-8.6), HER2/Osajin(-6.8), HER2/ CD66d(-8.9) and PDF/wighteone(-8.4) respectively with noticeable binding energy. Among the iso-flavanones alpinumisoflavone has several metabolic functions. Prior Chemical-Target interactions have linked it to a number of signaling and apoptotic molecules, including STAT6, BAX, and CASP3 (https://ctdbase.org/assessed). Furthermore, research implicate them to apoptotic cell death and cancer (Woonghee Lee et al., 2025; Tati Herlina et al. 2019). Osajin is another isoflavone, and prior Chemical-Target interactions have linked it to a number of cellular and tumor markers, including COX1, EGF, ESR2, FGF1, and CD44 (https://ctdbase.org/ evaluated on). Research indicates that the compound has anti-cancer properties (Shih-Yin Huang 2019; Tsung-Teng Huang et al., 2011). According to Chemical-Target interactions, weighteone an isoflavanones interact with a number of tumor and biological indicators, including AKT1, BCL2, and MAPK1 (https://ctdbase.org/ evaluated on). Research suggests that the chemical has anti-proliferative properties (Xiaofei Chen et al., 2024). According to the literature and chemical interactions mentioned above, these chemicals have significant roles in controlling cancer through a variety of biological pathways. Cumulatively, these lines of research suggests further in depth-research and extensive simulations are important confirm to unravel the potential of the phytoconstituents of *Erythrina variegate* in breast cancer treatment.

**Conclusion:** Based on cellular tests, including the MTT assay, apoptosis, and cell-cycle, the methanolic leaf extract of *Erythrina indica* demonstarted anti-cancerous activities. Changes in the gene expression of the apoptotic markers *Cmyc*, *P53*, and *Caspase 3* supplemented the findings. The best fit results for chemical-ligand conjugate was eEF-2K/alpinumisoflavone, ERα/alpinumisoflavone, HER2/Osajin, HER2/CD66d, and PDF/wighteone, respectively through docking simulations. Cumulatively the results implicate that the natural compounds have potential in breast cancer therapy given their roles in regulating cancer through various cellular mechanisms warranting future in detail studies.

**ETHICAL APPROVAL**

**DATA AVAILABILITY-**All data generated and analyzed are included within this article.

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This study does not involve experiments on animals or human subjects.

Tables-1-Details of drug treatment concentrations and respective cell lines

|  |  |  |  |
| --- | --- | --- | --- |
| **Sl. No.** | **Culture conditions** | **Cell lines** | **Concentration treated to cells** |
| 1 | Untreated | MCF7 | No treatment |
| 2 | Blank | - | Only Media without cells |
| 3 | Doxorubicin | MCF7 | 1 μM/mL |
| 4 | Methanolic extract | MCF7 | 25, 50, 100, 200, 400 μg/mL |

Table-2.Primer sequences for RT-PCR of apoptotic genes

|  |  |  |
| --- | --- | --- |
| Sl.no | Gene | Primer sequence |
| 1 | **P53** | Forward Sequence 5′- CCTCAGCATCTTATCCGAGTGG-3′Reverse Sequence 5′- TGGATGGTGGTACAGTCAGAGC -3′  |
| 2 | **Casp3** | Forward Sequence 5′- CACAAGCAGAGTGCTGAAGGTG-3′Reverse Sequence 5′- GATTCCTGAGAGTCCAAAGACAG-3′  |
| 3 | **cMyb** | Forward Sequence 5′- TGGCAAGCACTACCTGGATCAG-3′Reverse Sequence 5′- GCAGAGACTGTAGGTAGTTTCGG-3′  |
| 4 | **Beta actin** | Forward Sequence 5′- TCACCATGGATGATGATATCGC -3′Reverse Sequence 5′- ATAGGAATCCTTCTGACCCATGC-3′ |

Table-3 : Cell viability values (%)of Methanolic extract treated on MCF7 cells 24hrs post treatment.

|  |  |  |
| --- | --- | --- |
| **Condition** | **%cell viability** | **IC50 conc.(μg/mL)** |
| **Untreated** | 100 | 85.27 |
| **Dox- 1μM** | 51.64 |
| **ME extract-25 μg** | 87.79 |
| **ME extract-50 μg** | 65.81 |
| **ME extract-100 μg** | 42.92 |
| **ME extract-200 μg** | 21.44 |
| **ME extract-400 μg** | 8.66 |

Table 4: Cells (%) undergone Apoptosis, Necrosis in treated and Untreated MCF7 cells

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Quadrant** | **%Necrotic cells** | **%Late apoptotic cells** | **%Viable cells** | **% Early apoptotic cells** |
| **Label** | UL | UR | LL | LR |
| **Untreated** | 0 | 0.36 | 99.3 | 0.34 |
| **Std control** | 1.64 | 42.77 | 42.13 | 13.46 |
| **Methanolic extract** | 3.6 | 42.29 | 47.03 | 7.08 |

Table5 : The cells ( % ) arrested in the different phases of MCF7 cell cycle.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sl .No.** | Cell Cycle stage | Untreated  | Doxorubicin | Methanolic extract |
| **1** | Sub G0/G1 | 1.69 | 7.68 | 14.38 |
| **2** | G0/G1 | 56.13 | 48.26 | 49.81 |
| **3** | S | 5.09 | 4.01 | 3.79 |
| **4** | G2/M | 37.09 | 40.05 | 32.02 |

Table 6: Results of the molecular docking of various compounds

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **S.No.** | **Phytoconstituents/Target** | **CD66d** | **eEF-2K** | **ERα** | **PDF** | **HER2** |
| **1** | **Alpinumisoflavone** | -6.2 | **-8.7\*** | **-8.6\*** | -7.9 | -8.3 |
| **2** | **Beta Sitosterol** | -5.1 | -5.4 | -6.3 | -5.0 | -7.0 |
| **3** | **Caffeic acid** | -4.4 | -6.0 | -6.0 | -6.5 | -6.3 |
| **4** | **Docosanol** | -1.3 | -3.4 | -2.8 | -2.8 | -4.6 |
| **5** | **Erybidine** | -4.8 | -6.7 | -6.3 | -6.0 | -7.4 |
| **6** | **Erysodienone** | -5.8 | -6.5 | -5.5 | -6.5 | -7.5 |
| **7** | **Erysodine** | -4.5 | -6.2 | -5.8 | -6.2 | -7.2 |
| **8** | **Erysonine** | -5.2 | -7.2 | -6.4 | -5.5 | -7.3 |
| **9** | **Erysopine** | -5.3 | -6.9 | -6.0 | -5.8 | -7.5 |
| **10** | **Erysopitine** | -4.5 | -7.0 | -5.8 | -5.5 | -6.9 |
| **11** | **Erysotine** | -5.1 | -6.4 | -6.2 | -6.4 | -7.3 |
| **12** | **Erysotrine** | -5.1 | -6.0 | -6.2 | -7.1 | -5.9 |
| **13** | **Erysovine** | -4.8 | -6.5 | -6.2 | -6.1 | -6.7 |
|  **14** | **Erythraline** | -4.8 | -6.7 | -6.3 | -6.0 | -7.3 |
| **15** | **Erythrartine** | -5.2 | -6.1 | -5.6 | -7.0 | -6.2 |
| **16** | **Erythratine** | -5.8 | -7.1 | -6.4 | -6.1 | -7.1 |
| **17** | **Erythrinine** | -5.5 | -6.7 | -6.3 | -6.2 | -7.2 |
| **18** | **Erythromotidienone** | -5.4 | -6.9 | -7.6 | -6.2 | -7.6 |
| **19** | **Erythrosotidienone** | -5.2 | -7.6 | -7.2 | -6.6 | -7.8 |
| **20** | **Ferulic acid** | -4.6 | -5.3 | -5.4 | -5.5 | -5.7 |
| **21** | **Hypaphorine** | -4.1 | -5.4 | -4.5 | -4.9 | -4.9 |
| **22** | **Nororientaline** | -5.0 | -6.1 | 5.9 | -6.0 | -6.0 |
| **23** | **Osajin** | **-6.8\*** | -8.4 | -7.5 | -7.6 | **-8.9\*** |
| **24** | **Oxyresveratrol** | -5.1 | -6.7 | -6.9 | -5.3 | -7.1 |
| **25** | **Quercetin** | -5.3 | -7.6 | -7.2 | -6.8 | -8.3 |
| **26** | **Stigmasterol** | -5.4 | -6.7 | -5.5 | -5.6 | -7.2 |
| **27** | **Wighteone** | -5.8 | -8.1 | -7.2 | **-8.4\*** | -7.4 |

Bolded and asterix indicate- The best docking scores



Figure-1: Graphical representation of cell viability values (%) of Methanolic extract treated MCF7 cells post 24hrsincubation Period.



Figure-2: Quadrangular plots of Cell viability values% of Methanolic extract treated MCF7 cells post 24hrsincubation period analyzed by Flow cytometry.

Figure-3: Graphical representation of apoptotic cells (% ) in treated and untreated conditions.

Figure-4: Graphical representation of cells arrested (% ) or distributed in the different phases of MCF7 cell-cycle post treatment with Doxorubicin and Methanolic extract in comparison to control.

Figure-5: Relative mRNA expression of *P53*, *Caspase 3, c-Myb* genes by RTqPCR method in MCF7 cells in different culture groups viz., Untreated, Doxorubicin (concentration of 1uM ) and Methanolic extract with 85ug/ml concentration.



Figure-6: 3D ribbon structure depicting interaction of CD66d with Osajin(Arrow indicate bound chemical compound )



Figure-7: 3D ribbon structure depicting interaction of eEF-2k with Alpinumisoflavone. (Arrow indicate bound chemical compound )



Figure-8: 3D ribbon structure depicting interaction of ESR with Alpinumisoflavone (Arrow indicate bound chemical compound )



Figure-9: 3D ribbon structure depicting interaction of PDF with Wighteone (Arrow indicate bound chemical compound )