RESEARCH ARTICLE

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In Silico and *In Vitro* Evaluation of Novel Small Molecule Inhibitors Targeting Apoptosis Pathways in Breast Cancer Cells

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Abstract

Background: Breast cancer is one of the leading causes of cancer-related mortality globally. Recent advances in targeted therapies have focused on selectively inhibiting signalling pathways that are vital for cancer cell survival and proliferation. Identifying novel small molecules with such inhibitory capabilities remains a critical step toward more effective treatments. **Methods:** A computational approach was utilized to design and identify small molecule inhibitors targeting key signalling pathways implicated in breast cancer progression. Virtual screening and molecular docking analyses were conducted to identify potential lead compounds. The selected compounds were further evaluated *in vitro* using MCF7 breast cancer cells to determine cytotoxicity (via IC_{50}) and to assess apoptotic effects. ADMET profiling was performed to predict pharmacokinetic properties. **Results:** Two lead compounds, C12 and C18, demonstrated strong binding affinities to serine-threonine protein kinase and MAP3K5, both of which are critical regulators of apoptosis. In vitro studies revealed significant cytotoxic effects, with IC50 values of 22.49 ± 1.01 µg/ml for C12 and 14.61 ± 0.01 µg/ml for C18. Microscopic and biochemical analyses showed hallmark features of apoptosis, including nuclear condensation, DNA fragmentation, and mitochondrial membrane potential loss. ADMET predictions indicated favourable pharmacokinetic profiles for both compounds. **Conclusion:** The identified compounds C12 and C18 exhibit potent cytotoxic and pro-apoptotic activity in breast cancer cells, highlighting their potential as promising novel therapeutic agents. However, further in vivo studies are essential to validate their efficacy and safety profiles.

Keywords: Apoptosis- Breast cancer- MCF- 7- Molecular docking- ligand-protein interactions

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Introduction

Cancer has emerged as a significant health issue in the 21st century, with rising prevalence and far-reaching impacts on physical, mental, and social well-being [1]. The disease affects 1-2% of the population in developed countries, with a notable annual increase of nearly 5% in less developed nations [2]. Globally, cancer claims over 7 million lives annually, with projections indicating a rise from 10 to 15 million new cases [3]. Breast cancer is the most common malignancy among women worldwide, with over a million new cases annually. Despite India's lower age-adjusted incidence rate (25.8 per 100,000) compared to the UK (95 per 100,000), mortality rates are similar. Recent studies indicate rising cancer incidence and mortality in India, with breast cancer now surpassing cervical cancer as the leading cause of cancer-related deaths among Indian women [4].

Breast cancer has a complex etiology dating back to around 3500 BCE. It is characterised by uncontrolled cell growth in breast tissue, often originating in the milk-producing glands or ducts. Various types of breast cancer exist, including invasive ductal carcinoma, invasive lobular carcinoma, and ductal carcinoma in situ (DCIS). Several risk factors contribute to breast cancer development, including genetic mutations. Inherited mutations in genes like BRCA1 and BRCA2 significantly increase breast cancer risk, particularly among women aged 40-55. Hormonal influences, such as exposure to oestrogen and progesterone. Age factors that have been

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reported in women over 50 years. Also, family history has been studied as a significant risk factor, especially among first-degree relatives who have been diagnosed, with approximately 20% of breast cancer patients having a family history linked to a predisposing gene, highlighting the importance of genetic factors in breast cancer etiology [5], as represented in Figure 1. Lifestyle factors, such as alcohol consumption, lack of physical activity, and obesity, may contribute to breast cancer development. In addition, environmental factors such as exposure to radiation, certain chemicals, and viruses have also been linked to these risk factors.

A viewpoint may not resolve the risk factors of breast cancer; researchers have also suggested that viruses, such as human papillomavirus (HPV) and mouse mammary tumour virus (MMTV), might contribute to breast cancer development, although the exact mechanisms are still being studied [6].

Researchers continue to develop practical solutions for breast cancer; meanwhile, early treatments focused on localised approaches, including surgery and radiation. Historical milestones included radical Mastectomy, which William Halsted pioneered in the late 19th century, involving the removal of the breast, axillary lymph nodes, and chest muscles. The next progress was reported in radiation therapy, which was introduced by Marie Curie in 1898 [7]. Radiation induces DNA damage via ionising radiation (IR) or reactive oxygen species (ROS), leading to oxidative stress and DNA damage in cancer cells. Then, the 1960s marked a significant shift in breast cancer treatment, focused on systemic interventions driven by a deeper understanding of the disease. Systemic interventions, including hormonal, chemotherapy, and biological therapies, were introduced, expanding treatment options beyond localised approaches.

While chemotherapy uses common active agents like Anthracyclines (e.g., doxorubicin), Taxanes (e.g., paclitaxel), Fluoropyrimidines (e.g., 5-fluorouracil), and Platinum agents (e.g., carboplatin), anti-cancer drugs target and kill rapidly dividing cancer cells, shrinking or eliminating tumours, controlling disease progression, and relieving symptoms [8, 9]. Biological therapies, which focus on *HER2*-targeted therapies: Trastuzumab (Herceptin), a monoclonal antibody, improve outcomes in *HER2*-positive breast cancer patients when combined with chemotherapy. Next-generation *HER2*-targeted therapies include Lapatinib (tyrosine kinase inhibitor) and Pertuzumab (monoclonal antibody). These therapies have significantly improved treatment outcomes, reducing recurrence and mortality rates in *HER2*-positive breast cancer patients. Despite significant advances in diagnosis and treatment, developing targeted therapies that selectively inhibit signalling pathways crucial for cancer cell survival and proliferation is an area of ongoing research. The heterogeneity of breast cancer, characterised by diverse molecular subtypes, underscores the need for novel therapeutic strategies that can effectively target specific pathways involved in tumorigenesis.

This can be strategically achieved by predicting biological interactions between potential chemotherapeutic agents and biomarkers using *in silico* methods. This approach offers significant benefits, like optimising drug development, reducing costs, and streamlining clinical trials, ultimately bringing effective treatments to market faster. Hence, by harnessing computational power, researchers can enhance the efficiency and effectiveness of cancer treatment development.

Discovering Anti-Cancer Drugs via Computational Methods

Despite significant advancements in biotechnologies and a better understanding of disease biology, developing new, practical, and innovative small molecule drugs remains a complicated, time-consuming, and expensive project that necessitates collaborations from many experts in multidisciplinary fields such as medicinal chemistry, computational chemistry, biology, drug metabolism, clinical research, and so on. Furthermore, the effective discovery and development of a new treatment takes 12 years, a significant investment. Novel drug development

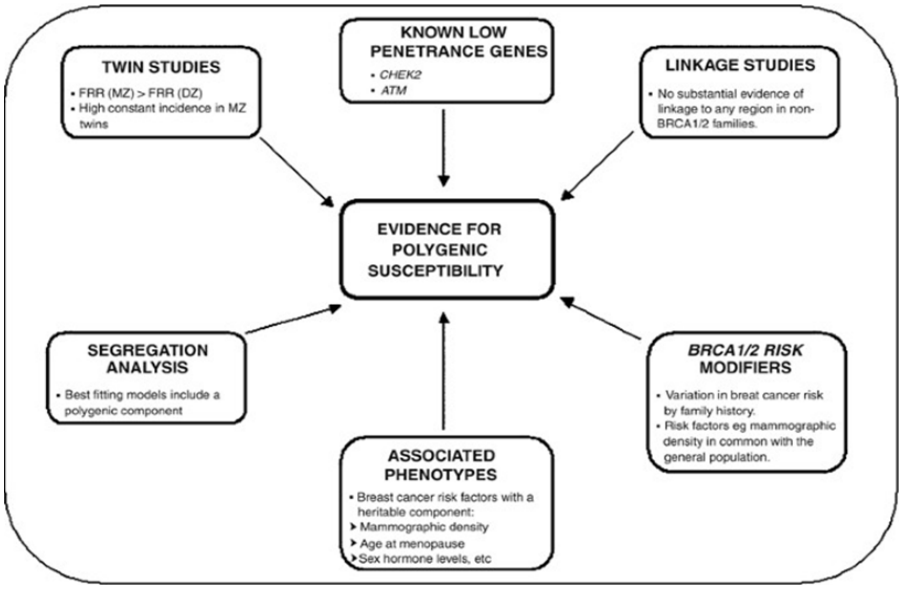

Figure 1. Evidence for Polygenic Susceptibility

procedures with lower time and money costs and increased efficiency are in high demand and could help to improve global health and life expectancy. Computational methods have been an essential tool in drug discovery projects and a cornerstone for novel drug development methodologies since the successful creation of HIV protease inhibitor Viracept in the United States in 1997, which was the first drug design wholly driven by its target structure. This speeds up and lowers the cost of drug development. Recent developments in computational capacity, especially massively parallel computing on graphics processing units (GPUs), and AI technologies have converted fundamental research into practical applications in the drug discovery area. Their excellent success in presenting fresh potential ideas and solutions to tackle life-threatening diseases drew much attention [10, 11].

Anti-Cancer Drug Target Prediction

Despite the vast number of potential pharmaceutical targets (~6,000-8,000 sites) among human genes, only about 400 encoded proteins have been successfully utilised in medication development. Cancer offers numerous potential therapeutic targets, but traditional drug development often focuses on single targets, overlooking complex disease mechanisms and polypharmacological effects. In addition to these limitations of the current approaches, Undruggable targets with certain proteins, such as phosphatases, transcription factors, and RAS family members, lack efficient, enzymatically active sites, making them challenging to target. Off-target effects are another unintended interaction that can lead to side effects, particularly in cancer medications. In addition, unknown targets associated with many drugs, including anticancer treatments, have unknown or uncharacterised target proteins. However, with innovative opportunities for drug target prediction and repositioning, identifying new indications for existing medicines can accelerate drug development. Bioinformatic target prediction can also facilitate high-quality methodologies that are essential for reliable drug target prediction and discovery. By addressing these challenges and leveraging innovative approaches, researchers can uncover new therapeutic targets and develop more effective treatments.

Various interactive web servers and databases, such as SEA (htts://omictools.com/sea-2-tool), Pharmmapper (htts://www.lilab-ecust.cn/pharmmapper/), SwissTarget Prediction (htts://www.swisstargetprediction.ch/), DrugBank (https://www.drugbank.ca/), ChEMBL(http://www.ebi.ac.uk/chembldb), and BindingDB (http://www.bindingdb.org/), amongst others, have been developed to facilitate drug target prediction. These resources provide valuable computational models and tools for researchers. Several computational models have been employed to investigate drug-protein interactions, including network-based models that analyse relationships between drugs, targets, and biological networks. Machine learning-based models, as well, which utilise supervised learning, bipartite graph learning, and other approaches to predict drug-target interactions.

These tools provide notable studies, such as side effect similarity analysis. For instance, one of the authors used side effect similarity to identify potential drug-target interactions. In another study, the author leveraged the FDA's AERS to predict large-scale drug-target interactions, which remains a crucial adverse event reporting system. In addition, recent studies have shown that deep learning, particularly neural networks, can outperform other methods in predicting drug targets [12]. These computational approaches have advanced the field of drug target prediction, enabling researchers to identify potential therapeutic targets and develop more effective treatments.

Ligand-Based Drug Discovery

A) Similarity searching

Ligand-based drug development relies on the concept of molecular similarity, where structurally similar molecules tend to exhibit similar biological effects. This approach uses structural information of active ligands to identify and predict new chemical entities with similar properties. It's an indirect protocol that doesn't require knowledge of the target protein's 3D structure, making it useful when the structure is unknown or unpredictable. Molecular descriptors, such as physicochemical parameters and 2D/3D similarity searches, are used to represent reference molecules. Hence, this method is frequently used to screen novel ligands with intriguing biological activities *in silico* and to optimise the biological activities of ligands to improve drug pharmacokinetics, including ADMET characteristics (Adsorption, Distribution, Metabolism, Excretion, Toxicity).

Molecular descriptors provide the basis for this simple and frequently utilised approach. To represent the reference molecules, physicochemical parameters (e.g., molecular weight, logP, Energy of highest occupied molecular orbital (EHOMO), Energy of lowest unoccupied molecular orbital (ELUMO), charges) as well as 2D fingerprint and 3D shape-similarity searches can be used as coordinates. For molecular representation in virtual screening, the 2D fingerprint (Molprint2D and Unity 2D) and 3D shape similarity approaches (MACCS), extended-connectivity fingerprints (ECFP), rapid overlay of chemical structures (ROCS), and Phase Shape are more commonly utilised. For example, Bologa et al. (2006) used 2D fingerprint and 3D shape-similarity approaches to find new agonists for the GPR30 receptor of the estradiol receptor family. Furthermore, both methods have been successfully used in virtual screens, and both technologies have outperformed docking methods in terms of scalability and computing time against various targets. The fundamental issue with such approaches is their preference for input molecules and difficulty determining which input structures to utilise [13].

B) Ligand-Based Pharmacophore Mapping

The pharmacophore-based technique, which develops a pharmacophore model (PH4) based on a collection of active substances, is another more precise approach than molecular descriptors. A pharmacophore is "a set of spatial and electronic features necessary to achieve effective supramolecular interactions with specific biological targets and to initiate (or stop) their biological

responses," according to the IUPAC. As a result, the most likely chemical properties are represented by the structural overlap of essential molecular features generated from active chemicals or a binding site in space. The newly discovered compounds that fit and complement the designed pharmacophore will likely be active against the desired target protein. As a result, they can be chosen as candidates for further research. Without macromolecular structures, this approach has become a fundamental computational strategy for promoting and guiding drug discovery.

The process of pharmacophore modelling can be summarised as follows

(i) Selection of a training set of ligands (active and inactive compounds).

(ii) Molecular preparation (low-energy conformations).

(iii) Ligand alignment/superimposition and pharmacophore model generation.

(iv) Validation of pharmacophore models.

The availability of a suitable training set of compounds exhibiting the same binding mode is critical for pharmacophore modelling based on ligands [14, 15].

Materials and Methods

Experimental

Materials and Instrumentation

All reagents used were of analytical grades and sourced from various suppliers: Ranbaxy Laboratories Ltd. (Mohali, India); E-Merck (India) Ltd. (Mumbai, India) for sodium bicarbonate and sulphanilic acids; Hi-Media Lab Pvt. Ltd (Mumbai) for DMEM, MEM, antibiotic solution, trypsin, and other chemicals; Acros Organics (New Jersey, USA) for MTT; Sigma Aldrich (Mumbai) for FBS, NBCS, PI, AO, and EB; S.D. Fine Chemicals Ltd. (Mumbai) is responsible for DMSO and paraformaldehyde, and Merck Pvt Ltd. (Mumbai) is responsible for glutaraldehyde. The MCF7 cell line was obtained from the National Centre for Cell Sciences, Pune, India. These epithelial cells were isolated from the breast tissue of a 69-year-old white woman with metastatic adenocarcinoma, making them suitable for breast cancer research. The cell line characteristics include human epithelial morphology, mammary gland tissue origin, and adenocarcinoma disease classification, with applications in 3D cell culture, immuno-oncology, anticancer activity, and cytotoxicity studies.

The following equipment were utilised: autoclave (Equitron), balance (Denver Instruments Apx 203), centrifuge (Remi R 24), CO2 incubator (NAPCO series 5400), deep freezer (-85°C, Krisp cold), ELISA microplate reader (Bio-Rad 550), filtration unit (Millipore), borosil glassware, hot air oven (American Universal), inverted microscope (Olympus IX 70), laminar airflow (Klenzaids), liquid nitrogen container (Cryocan BA 20), microtitre plates (Tarsons), Milli-Q water purification system (Millipore), pH meter (U Tech), water bath (NSW India), and gel electrophoresis system (Genie electrophoretic).

Methodology and Preparation

Preparation of Lead Compounds and Virtual Chemical Library

The 10 structurally diverse compounds of C1–C18 were selected to construct a virtual library of the lead compound. The chemical structure of all the compounds was prepared by ChemBioDraw Ultra Version 12.0. The conformational energies of the inhibitors have been reduced [16].

Drug Targets Prediction and Validation of Drug Targets

PharmMapper is a web server that uses pharmacophore mapping to identify potential drug targets. Capsaicin's molecular file was downloaded from the PubChem database (CID: 1548943) and uploaded to PharmMapper. The server generated conformations and mapped them to pharmacophore models in PharmTargetDB. Then, the N best-fitted hits with appropriate target annotations and aligned poses were listed. In addition, capsaicin's molecular file was submitted to DRAR-CPI and DDI-CPI servers for computational drug repositioning via chemical-protein interaction (CPI) analysis. All parameters were then set to default values [17–21].

Virtual Screening and Molecular Docking

Virtual screening techniques were deployed to identify the potential lead compounds from large databases. The pharmacophore features of the selected compounds were submitted to ZINCPharmer, a virtual screening software that searches over 176 million conformations [22]. This identified potential lead compounds. Molecular docking was then performed using PyRx 0.9 to understand ligand-receptor interactions. Bioinformatics tools and databases (PDB, PubChem, Marvin Sketch) were utilised in the research.

Preparation of Protein and Identification of Active Sites

By utilising the offline program protein data bank (PDB), the human VHL (PDB id.4W9H) with a resolution of 2.10 Å was obtained. The crystal water was removed from the protein (4W9H), followed by the addition of missing hydrogens, protonation, ionisation, and energy minimisation. The SPDBV (Swiss protein data bank viewer) force field was applied to minimise energy. The prepared protein was validated by utilising the Ramachandran plot. The amino acid residues present in the protein's active site were detected using Protein-ligand interaction profile (PLIP) https://plip-tool.biotec.tu-dresden.de/plip web/plip/index, an offline tool in Google.

Preparation of Ligands and Docking Analysis

A library of compounds was selected using ZINCPharmer and designed using Marvin Sketch. Docking analysis was conducted using PyRx 0.9 to identify top-scoring molecules, including a pyrazole analogue. Derivatives were designed and ranked based on binding affinity and energy values. The top 5 derivatives were selected based on binding energy values for further studies.

ADME, Toxicity, and Carcinogenicity Activity Predictions

Furthermore, the pharmacological and toxicological profiles of the compounds were evaluated via ADME (Absorption, Distribution, Metabolism, and Excretion), toxicity, and carcinogenicity properties of the designed pyrazole derivatives using:

1. SwissADME tool for ADME properties.
2. PreADMET online tool for toxicity and pharmacological properties.
3. CarcinoPred-EL for carcinogenicity prediction.

The study assessed various parameters, including Lipophilicity (logP value), blood-brain barrier (BBB) penetration, GI absorption, mutagenicity, and carcinogenicity.

In Vitro Cytotoxicity Assay

Determination of Cell Viability by MTT Assay

To provide a quantitative measure of cellular metabolic activity, the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to assess cell viability and cytotoxicity by the reduction of MTT by mitochondrial enzyme succinate dehydrogenase in living cells, resulting in the formation of a blue-coloured formazan product.

Briefly, exponentially growing cells (1×10^5cells/mL) were seeded in 96-well plates and treated with different concentrations in a 10–1000 µg/mL series for 24, 48, and 72 h with FCS-free complete medium. 100 µg of MTT (5 mg/mL) was added to 24, 48, and 72 h treated wells. After the plates were incubated at 37°C for 4 h, the supernatant was aspirated, and 200 µl of DMSO was added to each well to dissolve the Formozan crystals. Absorbance was measured at 540 nm using a 96-well microplate reader [23, 24].

HOECHST 33342 Staining for Nuclear Apoptosis

The MCF7 was seeded in 6-well plates and maintained at 37°C with 5% CO_2 in a humidified CO2 incubator for 48 h Subsequently, the cells were treated with C12 and C18 at their IC_{50} concentrations obtained in various incubation durations, such as 24 h, 48 h and 72 h, which were selected for this staining. At the indicated times, the medium was removed gently, and the cells were washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 20 min, re-washed, and stained with HOECHST 33342 (10µg/mL) at 37°C for 20 min in the dark. Stains were washed with methanol, followed by PBS, and the plate was immediately observed in the blue channel fluorescence with fluorescent microscopy [25].

Direct Fluorescence Microscopic Analysis for Apoptosis Induction by AO/EtBr

1µl of EtBr dye mixture (100 mg/ml acridine orange (AO) and 100 mg/ml ethidium bromide (EtBr), in distilled water) was directly stained with C12-treated cells grown on clean microscope cover slips. After staining, the cancer cells were washed with PBS (pH 7.2) and incubated for 1 min. The cells were then visualised under a fluorescence microscope at 400X magnification with an excitation filter at 480 nm [25].

Analysis of Mitochondrial Membrane Potential (ΔΨm) by Rhodamine 123 Staining

MCF-7 cells were seeded in 6-well plates (1×10^5cells/well) and allowed to grow for a day before exposure to IC_{50} concentrations of C12 and C18. After the specific time intervals (24, 48, and 72 h), the cells were fixed in 4% paraformaldehyde, washed twice with PBS, and exposed to the ΔΨm-specific stain Rhodamine 123 (Rh-123) (10 µg/ml) for 30 min at 37°C. The cells were washed twice with methanol to remove the excess stain, rewashed with PBS, and analysed for changes in ΔΨm using a fluorescence microscope with an excitation and emission wavelengths of 505 nm and 534 nm, respectively [25].

Measurement of DNA Damage by DNA Fragmentation

DNA damage by apoptosis was evaluated by genomic DNA fragmentation. The cells (1×10^6 cells) were separately suspended in 10 mL of buffer containing 10 mm Tris HCl and 10 mm EDTA (pH 8.0). The cells were treated with C12 fractions in 10 ml solution containing 10 mm Tris HCl, 10 mm EDTA (pH 8.0), and 20 mg/ml proteinase K. The mixture was incubated at 37°C for 3 h, followed by DNA extraction with phenol:chloroform: isoamyl alcohol solution (25:24:1). The extracted DNA was treated with DNase free RNase at a concentration of 20 mg/ml at 4°C for 45 min and precipitated with 100 ml of 2.5 M sodium acetate and 3 volumes of ethanol. The DNA fragmentation analysis was then carried out by electrophoresis using 10 µg of the extracted DNA from the selected cancer cells for a period of 45 min at 100 V on a 2% agarose gel containing ethidium bromide and visualised under the Gel Doc System [25].

Analysis of the Potential Pathway/Interaction Effects of Chemical Mixture

The ChemDIS-Mixture tool was used to identify the potential effects and mechanisms of coexposure to multiple chemicals. The tool integrates multiple databases (STITCH, Gene Ontology, KEGG, Reactome, SMPDB, Disease Ontology). Then, chemical-protein interactions, GO terms, pathways, and disease associations are analysed. The joint p-value for prioritisation were visualised using Venn diagrams. With the ChemDIS-Mixture, insights into the complex interactions between multiple chemicals and biological systems are predicted [26].

Results

Identification of the Potential Drug Target Using Pharmacophore Mapping Approach

The tool identified the target proteins for 20 compounds C1-C20. It compared the pharmacophores of the most active compounds with the built database of pharmacophore models. It provided the target information of 300 proteins, including their fitness score, number of pharmacophoric features, indication, and importance of each protein. The 300 proteins retrieved were ranked according to their fitness score. The top 10 proteins with fitness scores over 5.0 were studied to identify the possible target protein of all the compounds, and target selection was done based on the importance of the protein in cancer

Table 1. C12 - Prediction of Drug Target using Pharmacophore Mapping Approach

Ranks	PDB ID	Target Name	Features	Fit Score	Normalized Fit Score	z'-score
1	4O75	Serine-threonine protein kinase	3	3.761	0.8245	0.301238
2	2PE2	3-phosphoinositide-dependent protein kinase 1	5	2.474	0.7523	3.34059
3	2WI1	Orally Active 2-Amino Thienopyrimidine Inhibitors of the Hsp90 Chaperone	4	2.815	0.7037	1.24798
4	1CA7	Macrophage migration inhibitory factor	4	2.806	0.7016	1.22039
5	2CLX	Cell division protein kinase 2	4	2.777	0.6941	1.15494
6	1RTK	Complement factor B	4	2.765	0.6912	1.02773
7	2RKU	Serine/threonine-protein kinase PLK1	4	2.71	0.6776	0.915384
8	2BKZ	Cyclin-A2	4	2.565	0.6412	0.291971
9	2ZKC	Estrogen-related receptor gamma	4	2.421	0.6053	-0.163952
10	1XDC	Superoxide dismutase [Mn], mitochondrial	5	2.951	0.5902	1.65023

Table 2. C12 - Pharmacophore Modelling

Hydrophobic	Positive	Negative	Donor	Acceptor	Aromatic
1	0	0	0	2	0

disease (Tables 1, 2). Several proteins from Table 2 scored high fitness scores for C12 but weren't directly linked to diseases. However, Serine-threonine protein kinase AKT1, with a fitness score of 3.761, plays a crucial role in cancer. Growth factors activate AKT1 and promote cell survival by inhibiting apoptosis. It's also involved in growth-promoting signals and tumour growth. Notably, AKT1 deficiency can lead to cancer resistance and growth delays. A specific genetic variation in AKT1 can cause Proteus syndrome. The graphical pharmacophore models of C-12 and C-18 are represented in Figures 2- 5, respectively.

The protein MAP3K5 (ASK1) scored a high fitness score of 3.991 for C-18 and plays a crucial role in inducing apoptosis, as recorded in Table 3, with its pharmacophoric corresponding values in Table 4. ASK1 is a key regulator of cellular stress response, activating JNK and p38 pathways. It's implicated in various diseases, including cancer, diabetes, and neurodegenerative disorders. ASK1's activity is tightly regulated by proteins like thioredoxin and CIB1, which inhibit its function under normal conditions. Upon stress, ASK1 is activated through a complex mechanism involving TRAF2 and TRAF6, leading to its full activation and induction of apoptosis [2]. ASK1 expression is regulated at multiple levels: inflammatory cytokines like IL-1 and TNF-α can induce its transcription, while TNF-α also stabilises the ASK1 protein by preventing its degradation. This unique dual regulation sets ASK1 apart from other MAP kinase family members [13].

Virtual Screening and Docking

In PyRx, binding affinity parameters were considered for selecting the best "HITS" and compared with the co-crystal. PyRx binding energy is the interaction energy between the protein and the ligand. This RMSD value strongly indicates the extent of the interaction of proteins and ligands. The compounds whose binding energy was above standard (co-crystal) are shown in Figures 4-7 and Table 5, indicating that the compounds were effectively bound to the active site of proteins (6HHG and 4O75). The interactions of these proteins and these compounds were analysed.

ADMET Analysis and Drug-Likeness Analysis

The ADMET properties and drug-likeness properties of all selected compounds were predicted. The pre-ADMET analysis predicts that two compounds have medium to high blood-brain barrier (BBB) penetration

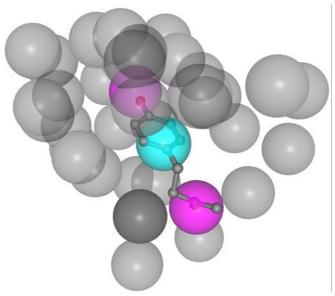

Figure 2. Molecule and Pharmacophore Model for C12

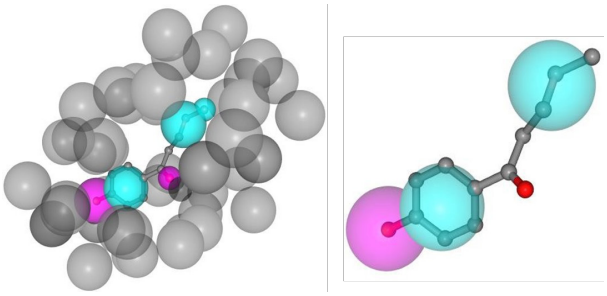

Figure 3. C18 - Molecule and Pharmacophore Model

Table 3. C18 - Prediction of Drug Target using Pharmacophore Mapping Approach

Ranks	PDB ID	Target Name	Features	Fit Score	Normalized Fit Score	z'-score
1	6HHG	MAP3K5	3	3.991	0.997	1.27722
2	1III	Transthyretin	3	2.97	0.9901	1.28169
3	2PIO	Androgen receptor	3	2.965	0.9883	1.21432
4	1BM6	Stromelysin-1	3	2.963	0.9877	1.16901
5	1PMV	Mitogen-activated protein kinase 10	3	2.929	0.9762	1.04632
6	1UKI	Mitogen-activated protein kinase 8	3	2.903	0.9676	0.959501
7	1YXX	Proto-oncogene serine/threonine-protein kinase Pim-1	3	2.893	0.9644	1.014
8	1PME	Mitogen-activated protein kinase 1	3	2.885	0.9615	0.929245
9	1RS0	Complement factor B	3	2.878	0.9595	0.914489
10	3EQM	Cytochrome P450 19A1	3	2.844	0.9481	0.824923

Table 4. C18 - Pharmacophore Colour Scheme

Hydrophobic	Positive	Negative	Donor	Acceptor	Aromatic
2	0	0	0	1	0

based on the Cbrain/Cblood ratio. Then, 18 of the 20 compounds were screened, and compounds C-12 and C-18 progressed for further testing. Hence, they were evaluated for drug-likeness, which assesses pharmacological and toxicity properties. The analysis revealed that all compounds met Lipinski's rule, with ≤5 hydrogen bond donors, <10 hydrogen bond acceptors, molecular weight <500, and ClogP <5. Compounds CH15, CH17, CH29, and CH47 showed strong binding affinity (<<0.1µm). Most compounds satisfied the CMC rule (no violations), the MDDR rule (value of 1), and the WDI rule (no violations). These results indicate favourable drug-like properties for the compounds. Also, CarcinoPred-EL was used to assess the carcinogenicity of two compounds. The results indicate no mutagenic activity in the Ames test (no point or frameshift mutations). No negative mutagenicity in a carcinoma mouse model and medium risk of hERG inhibition, potentially prolonging the QTc interval and increasing the risk of cardiac arrhythmias. These findings suggest the compounds may have a relatively safe carcinogenicity profile, but the hERG inhibition risk warrants further evaluation.

The corresponding outstanding ADMET, toxicity, and drug-likeness properties of C12 and C18 were statistically reported in Supplementary Tables 1-3. The compounds are firmly bound to plasma proteins and exhibit good GI absorption, making them suitable for oral dosage forms.

Carcinogenicity Activity of Compounds

Carcinogenicity is one of the cell-killing properties

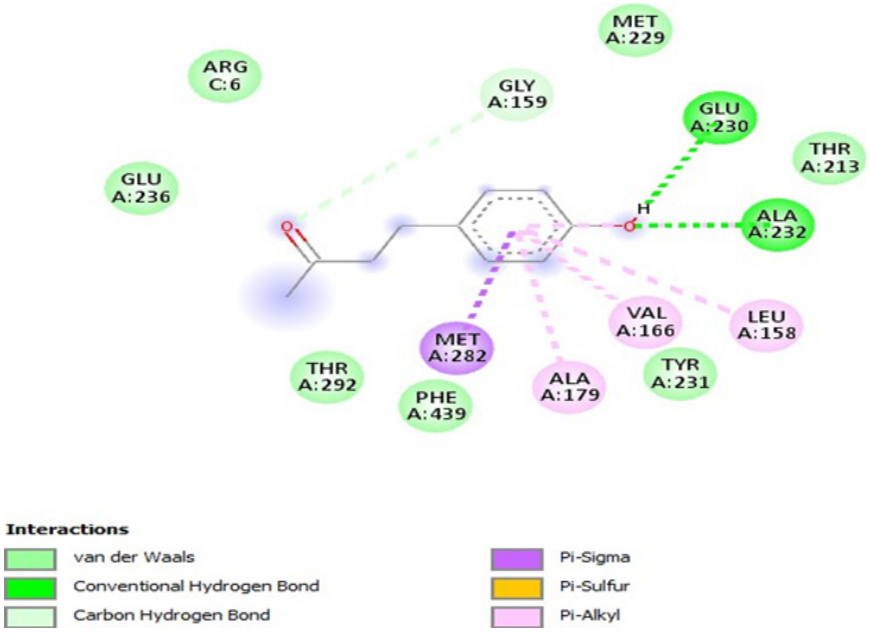

Figure 4. ligand: 4-(4- hydroxyphenyl) – 2 butanone, Protein: AKT1

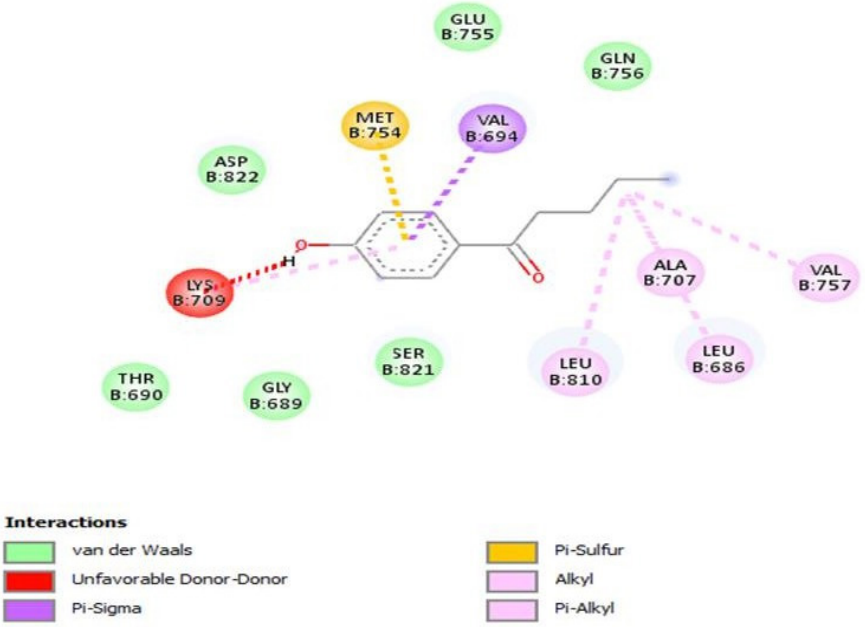

Figure 5. Ligand: 4-Hydroxyvalerophenone, Protein: MAP3K5

Table 5. Docking Results of the Selected Compounds

S. No.	Ligand	Protein	Binding Affinity
1	4-(4-Hydroxyphenyl)2- butanone	AKT1	-5.9
2	4-Hydroxyvalerophenone	MAP3K5	-6.6
3	Paclitaxel	AKT1	-7.9
		MAP3K5	-7.1

of the chemical compound. CarcinoPred-EL was used to determine the carcinogenic nature of the compound. The results showed that neither compound is mutagenic in the Ames test, either by point frameshift mutation (Supplementary Table 4), and none of the compounds showed negative mutagenicity on a carcinoma mouse model. Inhibition of these prolongs QTC intensity and the risk of cardiac arrhythmias. Both compounds showed a medium risk of hERG inhibition (Supplementary Table 4).

In vitro Cytotoxicity by MTT Assay

In vitro cytotoxicity using MTT was performed for the two compounds (C12 and C18). The authenticated cell morphology of MCF7 by MTCC is graphically

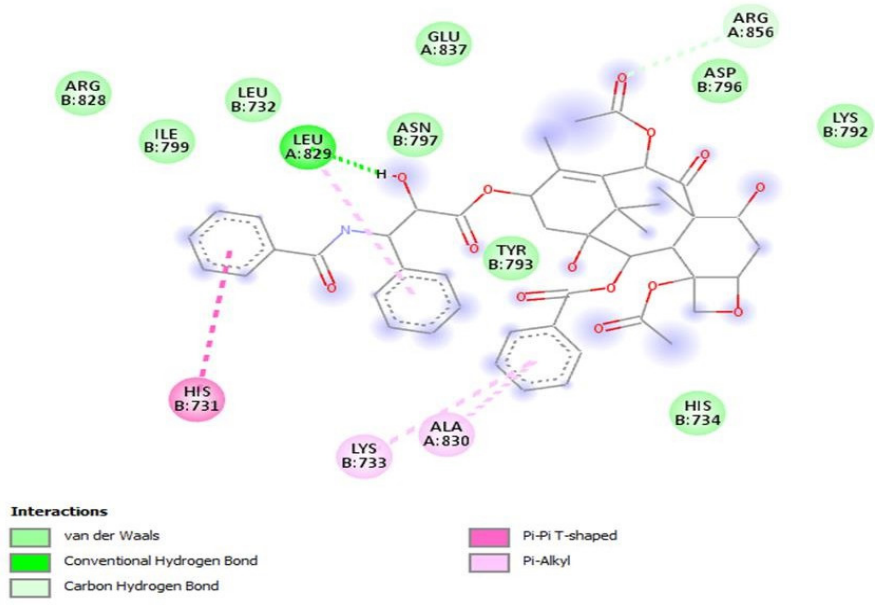

Figure 6. Ligand: PACLITAXEL(STD), Protein: AKT1

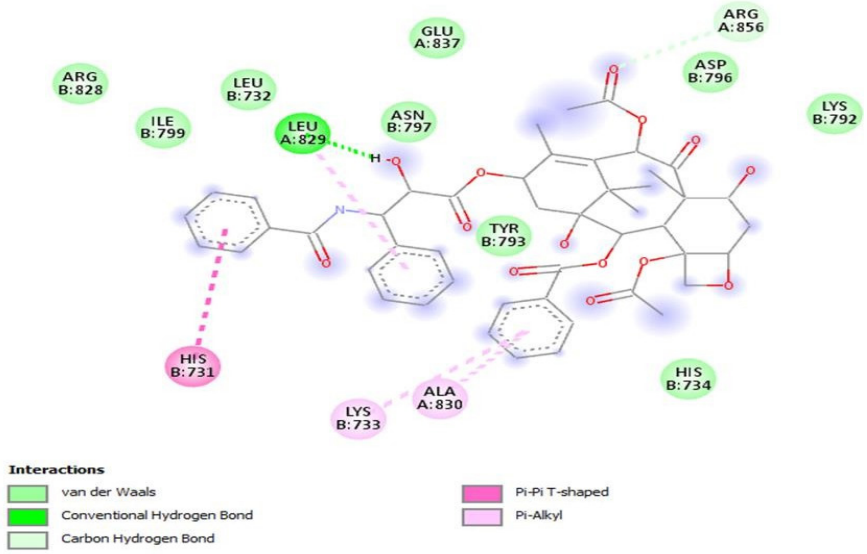

Figure 7. Ligand: PACLITAXEL(STD), Protein: MAP3K5

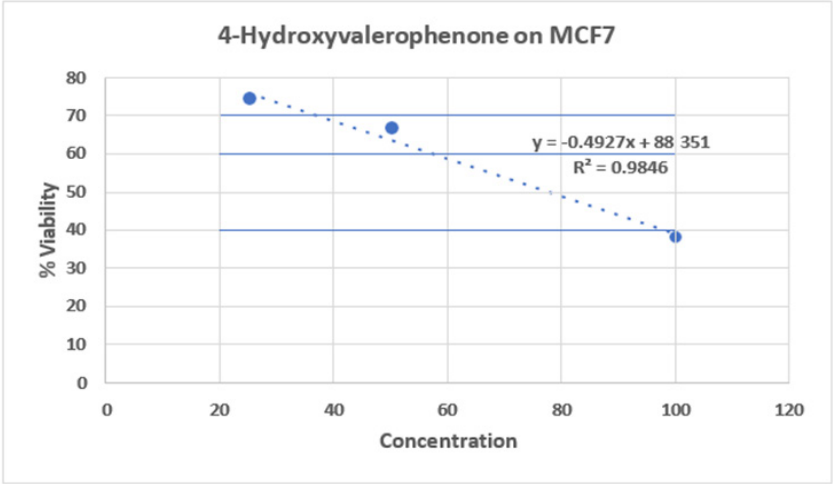

Figure 8. Cytotoxic Effects of Compound C12 on MCF7 Breast Cancer Cells as Determined by MTT assay. Cells were treated with varying concentrations of C12 for 72 hours, and cell viability was measured based on mitochondrial metabolic activity. IC_{50} value was calculated to assess cytotoxic potency.

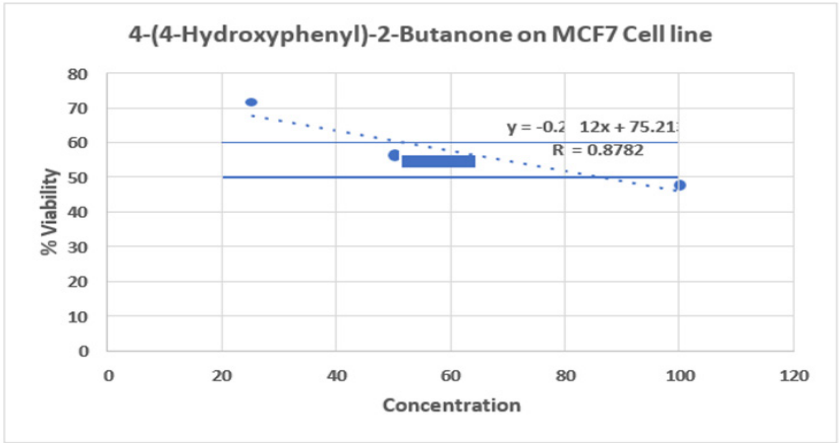

Figure 9. Cytotoxic Effects of Compound C18 on MCF7 Breast Cancer Cells as Determined by MTT assay. Cells were treated with varying concentrations of C18 for 72 hours, and cell viability was measured based on mitochondrial metabolic activity. IC_{50} value was calculated to assess cytotoxic potency.

represented in Figure S1. The IC_{50} value was calculated using the standard method. The percentage of Viability was calculated, and the percentage inhibition was calculated. The MCF7 cell line was used for the cytotoxicity study. 4-(4-Hydroxyphenyl)-2-Butanone showed IC_{50} value of 22.49±1.01 µg/ml and 4-Hydroxyvalerophenone showed IC_{50} value of 14.61±0.01 µg/ml respectively. This cytotoxic concentration will be used for further study in the MCF7 cell line (Figures 8 and 9).

Discussion

HOECHST 33342 Staining for Nuclear Apoptosis

The treatment with compounds C12 and C18 at CTC50 induced apoptosis in cancer cells. HOECHST 33342 staining revealed characteristic apoptotic features Figure S2a-b. These included reduced nuclear size, condensed chromatin, and nuclear fragmentation. The results suggest that growth inhibition (apoptotic nuclei of treated cells) was associated with apoptosis induction. C12 and C18's anti-proliferative effects are linked to their ability to induce apoptosis. Many authors observed the apoptosis of drug-treated cancer cells using morphological characteristics such as chromatin condensation, nuclear pyknosis, formation of apoptotic bodies, and atomic fragmentation using HOECHST. Therefore, the anti-proliferation effect of compounds C12 and C18 would be associated with their potential to induce apoptosis in the selected cancer cells.

Direct Fluorescence Analysis for Apoptosis Induction by AO/ EtBr

Cell morphological changes were observed using AO/EtBr fluorescence staining after treating them with compounds C12 and C18 at their CTC50 concentration. The result revealed that the apoptotic cells containing the condensed form of nuclei and apoptotic bodies were stained orange. Whereas the necrotic cells were stained red, the untreated MCF7 cells were stained uniformly green. Significant differences in apoptosis induction were observed between the control and MCF7 cells after treatment with C12 and C18 (Figure S3a and b). Clear apoptosis was detected in MCF7cells treated with C12 and C18.

Analysis of Mitochondrial Membrane Potential by Rhodamine 123 Staining

The mitochondrial membrane potential loss of MCF7 cells was analysed using the dye Rh-123, and a decrease in mean fluorescence intensity was observed following the treatment of cells with C12 and C18. The fluorescence images demonstrated the loss of mitochondrial membrane potential (Figure S4a and b) due to mitochondrial membrane depolarisation, an initial and irreversible step of apoptosis. The data indicated that the induction of apoptosis in cells by C12 was higher than that of C18, accompanied by alterations in the mitochondrial membrane potential. Besides, it was reported that mitochondria played an essential role in an intrinsic apoptotic pathway by releasing cytochrome c, leading to the activation of the caspase cascade. The results demonstrated that both C12 and C18 could disrupt the functions of mitochondria at the early stages of apoptosis, subsequently coordinating caspase three activation through the cleavage of caspases by the release of cytochrome c.

Determination of Apoptosis by DNA Fragmentation Assay

A DNA fragmentation assay was carried out to confirm the apoptosis induced by C12 and C18. We examined the nuclear DNA fragmentation by agarose gel electrophoresis, and the results are shown below. A DNA ladder pattern was observed after treating MCF7 cells with both compounds. This suggested that these C12 caused DNA fragmentation characteristic of the apoptotic process, with the generation of multiple DNA fragments, and induced apoptosis in these MCF7 cells at different incubation durations. A biochemical hallmark of apoptosis was the cleavage of chromatin into small pieces, including oligonucleosomes, which were described as DNA ladders in the electrophoresed gel Figure S5. DNA laddering was compared for apoptosis, and both compounds showed multiple bands. This confirms the apoptotic-inducing nature of C12 and C18.

In conclusion, this study employed a virtual screening approach to identify potential lead compounds and validate the compounds' *in vitro* activities against cancer targets, including serine-threonine protein kinase and MAP3K5, which play crucial roles in cancer and apoptosis. Molecular docking: PyPx docking showed strong binding affinities of compounds C12 and C18 to protein targets. ADMET analysis: Both compounds exhibited favourable pharmacokinetic properties, including high plasma protein binding and GI absorption. These potential lead compounds demonstrated cytotoxic effects against MCF7 breast cancer cells, with IC_{50} values of 22.49±1.01 µg/ml and 14.61±0.01 µg/ml, respectively. Apoptosis induction: Fluorescence microscopy and DNA fragmentation assays revealed characteristic apoptotic features, including nuclear condensation, fragmentation, and DNA laddering. These findings suggest that compounds C12 and C18 exhibit promising anticancer activity and merit further investigation for therapeutic development.

Author Contribution Statement

Study conception, design,: PK and RRK, analysis and interpretation: KM and VB, draft manuscript preparation: BWI, VB, KM. All authors reviewed and approved the final manuscript.

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If any scientific Body approved it/ if it is part of an approved student thesis

The work is part of the student thesis.

How the ethical issue was handled

The research work does not involve any ethical issues or ethical clearance.

Any conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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