SYNTHESIS, *IN SILICO* STUDIES AND CYTOTOXICITY ACTIVITY AGAINST BREAST CANCER CELLS OF NEW COUMARIN-BASED COMPOUNDS WITH CHALCONE AND PYRAZOLINE SCAFFOLDS

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

Rf	Retention factor
MHz	Mega Hertz
s	Singlet
d	Doublet
t	Triplet
m	Multiplet
cm^{-1}	per centimetre
ppm	parts per million
^oC	degree Celcius
δ	chemical shift
g	Gram
mL	Millilitre
µL	Microlitre
mg	Milligram
µg	Microgram
µM	Micromolar
J	coupling constant
rt	Room temperature
%	Percent
Å	Angstrom
ns	Nanoseconds

LIST OF ABBREVIATIONS

DMSO-d_6	Deuterated Dimethyl Sulfoxide
FT-IR	Fourier Transform Infrared
NMR	Nuclear Magnetic Resonance
^{13}C NMR	Carbon Nuclear Magnetic Resonance
^{1}H NMR	Hydrogen Nuclear Magnetic Resonance
EtOH	Ethanol
MeOH	Methanol
NaOH	Sodium Hydroxide
H_2NNH_2	Hydrazine hydrate
$H_2NNHCONH_2.HCl$	Semicarbazide hydrochloride
H_2NNHPh	Phenyl hydrazine
3ERT (ER-α)	Human Estrogen Receptor-alpha
ER+	Estrogen receptor-positive
Pdb	Protein data bank
Pdbqt	Protein data bank file specified for docking
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
MCF-7	Michigan Cancer Foundation-7 Cell Line
MTT	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)
L929	Mouse Fibroblast L929 Cell Line
IC_{50}	Half maximal inhibitory concentration
SI	Selectivity index

In vitro	Latin word meaning ″in a test tube″

LIST OF APPENDICES

SINTESIS, KAJIAN *IN SILICO* DAN AKTIVITI KETOKSIKAN TERHADAP SEL KANSER PAYUDARA BAGI SEBATIAN BAHARU BERASASKAN KUMARIN DENGAN PERANCAH KALKON DAN PIRAZOLINA

ABSTRAK

Sebatian berasaskan kumarin telah menarik perhatian utama kerana potensinya sebagai agen antikanser, terutamanya dalam rawatan kanser payudara. Matlamat kajian ini adalah untuk mereka bentuk sebatian baharu berasaskan kumarin dengan perancah kalkon dan pirazolina menggunakan kaedah pengedokan molekul, diikuti dengan sintesis dan pencirian sebatian menggunakan teknik spektroskopi FTIR dan NMR. Kaedah *in silico* SwissADME telah digunakan untuk menilai sifat farmakokinetik dan keserupaan dadah dan ujian *in vitro* telah dijalankan untuk menentukan potensi aktiviti mereka terhadap garisan sel kanser payudara MCF-7. Sejumlah 26 sebatian kumarin-kalkon telah disintesis melalui kondensasi Claisen-Schmidt, diikuti oleh tindak balas siklo-kondensasi bagi membentuk 36 sebatian kumarin-pirazolina. Beberapa sebatian kumarin-kalkon dan kumarin-pirazolina yang terpilih telah dilakukan saringan awal dengan menggunakan ujian radikal hapus sisa DPPH (2,2-difenil-1-pikrilhidrazil) bagi menilai sifat antioksidan sebatian tersebut sebagai panduan kepada penilaian sitotoksisiti terhadap garisan sel kanser payudara, MCF-7. Keputusan pengedokan molekul dengan 3ERT menunjukkan bahawa sebatian mematuhi aturan 5 Lipinski yang memaparkan ciri fisikokimia, farmakokinetik dan keserupaan dadah. Pengedokan semula sebatian **10, 10A,** dan **12** dengan BRCA-1 (4OFB) dan p53 (1YCR), menunjukkan tenaga pengikatan yang dipertingkatkan, interaksi dan konformasi stabil (RMSD < 2 Å) dalam simulasi dinamik molekul. Sebatian **10, 10A, 12,** dan **4i** menunjukkan keberkesanan yang ketara terhadap sel

kanser payudara manusia, MCF-7, dengan IC_{50} masing-masing, 6.44 ± 0.65 μM, 8.48 ± 1.06 μM, 9.03 ± 0.96 dan 11.57 ± 1.57 μM, yang mana telah menunjukkan aktiviti yang lebih baik daripada ubat rujukan, Tamoxifen (IC_{50} = 17.42 ± 1.48 μM). Tambahan pula, sebatian **10, 10A**, dan **12** menunjukkan nilai indeks selektiviti (SI), masing-masing iaitu 6.95, 5.27 dan 4.76, antara sel MCF-7 (kanser payudara) dan sel L-929 (fibroblas normal) yang menunjukkan ketoksikan selektif yang kuat terhadap sel kanser berbanding sel normal. Sebatian ini juga menunjukkan sifat farmakokinetik dan keserupaan dadah yang berpotensi, mendedahkan keupayaan mereka sebagai agen yang penting untuk pembangunan selanjutnya.

SYNTHESIS, *IN SILICO* STUDIES AND CYTOTOXICITY ACTIVITY AGAINST BREAST CANCER CELLS OF NEW COUMARIN-BASED COMPOUNDS WITH CHALCONE AND PYRAZOLINE SCAFFOLDS

ABSTRACT

Coumarin-based compounds have attracted significant attention for their potential as anticancer agents, particularly in the treatment of breast cancer. The aim of this study is to design new coumarin-based compounds with chalcone and pyrazoline scaffolds using the molecular docking method, followed by the synthesis and characterisation of the compounds using FTIR and NMR spectroscopic techniques. The *in silico* methods of the SwissADME were used to evaluate their pharmacokinetic and drug-likeness properties and the *in vitro* assay was carried out to determine their potential activities against MCF-7 breast cancer cell lines. A total of 26 coumarin-chalcone compounds were synthesised via the Claisen-Schmidt condensation, followed by their cyclo-condensation reaction to form 36 coumarin-pyrazoline derivatives. Some selected coumarin-chalcone and their corresponding coumarin-pyrazoline compounds were subjected to a preliminary screening using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay to evaluate their antioxidant properties to guide the selection of potential compounds for cytotoxicity evaluation against breast cancer cell lines, MCF-7. Molecular docking results with 3ERT indicated that the compounds obeyed Lipinski's rule of 5 demonstrating favourable physicochemical, pharmacokinetic and drug-likeness properties. The redocked compounds **10, 10A,** and **12** with BRCA-1 (4OFB) and p53 (1YCR), showed enhanced binding energies, interactions, and stable conformations (RMSD < 2 Å) in molecular dynamics simulations. Compounds **10, 10A, 12,** and **4i** displayed significant

efficacies against human breast cancer cells, MCF-7, with the IC_{50} of 6.44 ± 0.65 μM, 8.48 ± 1.06 μM, 9.03 ± 0.96 μM, and 11.57 ± 1.84 μM, respectively, which showed better activity than the reference drug Tamoxifen (IC_{50} = 17.42 ± 1.48 μM). Furthermore, compounds **10, 10A,** and **12** exhibited a selectivity index (SI) value of 6.95, 5.27 and 4.76, respectively, between MCF-7 (breast cancer) and L-929 (normal fibroblast) cells which indicates a strong selective cytotoxicity toward the cancer cells compared to the normal cells. These compounds also demonstrated promising pharmacokinetic and drug-like attributes, revealing their potentiality as promising agents for further development.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cancer ranks as the second most common cause of numerous deaths globally following cardiovascular diseases. Cancer is the unrestrained and swift growth of abnormal cells within the body. Through mitosis, the altered normal cells rapidly multiply, affecting nearby cells and eventually leading to tumor formation. The malignant, cancerous tumour has the capacity to invade and spread to nearby cells (Jemal et al., 2011; Syam et al., 2012; Salum et al., 2020). Worldwide cancer reports have shown that breast cancer is the most common type of cancer among women and over 70% of them are estrogen receptor α (ERα) positive (Komoto et al., 2018; Zhou & Liu, 2020). Estrogen, a hormone produced in the ovaries, plays a crucial role in the development and maintenance of female reproductive tissues, including the breasts and uterus, helping to regulate the menstrual cycles. It maintains healthy bones and cardiovascular health by regulating cholesterol levels and protecting against bone loss. However, prolonged exposure to estrogen increased risk of breast cancer (Al-Shami et al., 2023).

The biological effects of estrogen are regulated through estrogen receptors which play a key role in regulating gene expression and influencing cell growth and function. When estrogen binds to these receptors, the estrogen-receptor complex forms and enters the nucleus of the cell, where it interacts with DNA to regulate the transcription of specific genes (Paterni et al., 2014). The estrogen receptors (ERs), ERα (Estrogen Receptor Alpha) and ERβ (Estrogen Receptor Beta), are major prognostic markers used to identify and categorise breast tumors. They have different roles in mediating the effects of estrogen, with varying affinities for the hormone (Venkatesan

et al., 2010). ERα promotes cell proliferation, especially in breast tissue. When estrogen binds to ERα, it stimulates the growth and division of breast cells. ERβ, on the other hand, have anti-proliferative effects and counterbalance the actions of ERα in certain contexts (Akram et al., 2017). A huge number of drugs (ligands) have been investigated on their binding with ERα which is important in the genesis and evolution of hormone-dependent breast cancer (Bafna et al., 2020).

Selective estrogen receptor modulators (SERMs) are an important class of therapeutic agents with broad applications in the treatment of breast cancer. Their ability to act selectively on estrogen receptors provide the beneficial effects of estrogen in targeted tissues while minimising adverse effects (Patel & Bihani, 2018). Tamoxifen is the most widely used SERMs and serves as the standard adjuvant hormonal therapy for estrogen receptor-positive (ER+) breast cancer, which can minimise the risk of breast cancer reappearance and death (Yao et al., 2020). Tamoxifen has antagonist action and blocks the estrogen receptors (ER) in breast tissue, making it a commonly used treatment for estrogen receptor-positive (ER+) breast cancer. Since ER+ breast cancer cells rely on estrogen for growth and proliferation, tamoxifen effectively inhibits tumour growth and proliferation of cancer cells by preventing estrogen from activating these receptors (Criscitiello et al., 2011; Yao et al., 2020). In the study of estrogen receptor-positive (ER+) breast cancers, MCF-7 cells, derived from a human breast adenocarcinoma are widely used, making them an excellent model for investigating how estrogen influences breast cancer development and progression (Blakely et al., 2023). Since tamoxifen also has adverse effects, alternative treatment is needed and research in the area has been continuously developed.

Depending on the type of cancer and the stage at diagnosis, a variety of cancer treatments are available. If cancer has spread beyond its original location (a process

known as metastasis), systemic treatments such as chemotherapy are often required. Despite the availability of several anticancer agents currently in clinical practice, their effectiveness is often compromised by significant challenges, including severe side effects and the development of multidrug resistance. These factors limit the efficacy of treatment and highlight the need for more effective, targeted therapies (Kifle et al., 2021). Therefore, cancer treatment is based on the specific type of cancer, its nature, and its stage of development. The treatment strategy is tailored to whether the cancer is localised or has spread (metastasised), as well as other factors (Liu et al., 2024). However, these chemotherapeutic drugs have negative side effects that impair the quality of life for cancer patients by destroying healthy body cells and causing organ toxicity (Nurgali et al., 2018). Researchers are constantly driven to create safer, more effective, and selective anticancer medications due to their limitations and side effects. The search for novel chemotherapeutic drugs that target cancer cells while remaining safe for healthy cells has been ongoing (Montoya et al., 2021).

One of the important strategies in modern drug development is to explore the drug-receptor relationship through molecular docking. This computational technique is a powerful tool in modern pharmacology and cancer research, offering valuable insights in understanding how drugs (ligands) interact with their biological targets (such as proteins, nucleic acids, or lipids), which plays a pivotal role in structure-based drug design. It enhances the development of more effective and selective anticancer agents by predicting their interactions at a molecular level, which is essential for reducing side effects and overcoming challenges like drug resistance (Ferreira et al., 2015; Peters, 2018).

The use of natural product scaffolds in the design of new anticancer compounds is a promising area of research. These natural scaffolds provide unique chemical

diversity and biological activity, making them excellent starting points for drug design. By focusing on selectivity, solubility, stability, and minimising toxicity, researchers can harness the diverse biological activity of natural product compounds to create smaller, more effective, and safer anticancer drugs (Guo, 2017). Numerous plants have been exploited and many important constituents have been identified to exhibit inhibitory activities against various types of cancers with fewer side effects. The constituents such as phenolics, flavonoids, alkaloids, and terpenoids (Choudhari et al., 2020) have been chemically modified to develop new compounds with enhanced anticancer efficacies and safety profiles (Nasim et al., 2022). Natural and synthetic molecules with natural product scaffolds, such as coumarin, chalcone and pyrazoline rings showed diverse pharmacological activities (Rudrapal et al., 2021).

In designing new compounds, large and complex structures are unfavourable due to solubility, absorption, and metabolism problems. Hence, small molecules without unnecessary functional groups are preferred (Guo, 2017). The unique structures and properties of small molecules and their ability to interact with specific biological targets allows for the design of precise, selective therapies that minimise side effects and maximise therapeutic benefits. These advantages make them attractive for drug development (Wu et al., 2023).

Hybrid compounds, known as bifunctional or multifunctional molecules, are gaining attention in the field of drug discovery. By combining two or more active scaffolds into a single molecule, hybrid compounds will be more potent, selective, and safer therapeutics (Alkhzem et al., 2022). These hybrid molecules can exhibit enhanced pharmacological potential due to the synergistic effects of their scaffolds which can target multiple biological pathways or receptors, leading to improved efficacy, selectivity, and reduced side effects. The integration of different active

scaffolds in a molecule can improve the pharmacokinetics (absorption, distribution, metabolism, and excretion) of the drug, leading to better bioavailability and longer half-life (Shaveta et al., 2016; Singh & Kumar, 2023).

Coumarin is a heterocyclic compound and belongs to the benzo-α-pyrones class where the benzene ring is fused to the pyrone ring (Figure 1.1). Coumarin, known as *2H*-1-benzopyran-2-one or *2H*-chromen-2-one is a significant family of oxygen-containing heterocycles. It is a flavourful organic compound found in plants and naturally occurring polyphenolic. The term ″coumarin″ originates from the French word ″coumarou,″ which specifically refers to the ″tonka bean.″. In 1820, Vogel was first isolate coumarin from tonka beans from *Dipteryx odoranta* wild fruit of the fabaceae family (Tsivileva et al., 2022). Since then, the isolation synthesis, structural characterisation and the biological activities of coumarin were reported from plants, fungi, and bacteria. Coumarins play a significant role in both natural and artificial organic chemistry and are known for their diverse pharmacological properties (Stefanachi et al., 2018).

The coumarin scaffold is a key structure in the development of anticancer drugs, owing to its versatile biological properties. Numerous coumarin derivatives have demonstrated significant anticancer activity across various cancer cell lines, making them attractive candidates for drug development (Al-Warhi et al., 2020; Rawat & Reddy, 2022; Koley et al., 2024). Coumarin scaffolds can interact with enzymes and receptors via weak bond interactions, making them bioactive scaffold. Coumarins have shown the ability to inhibit cancer cell growth through various mechanisms such as inducing apoptosis, inhibiting angiogenesis, and modulating key cancer-related pathways (Akkol et al., 2020; Annunziata et al, 2020).

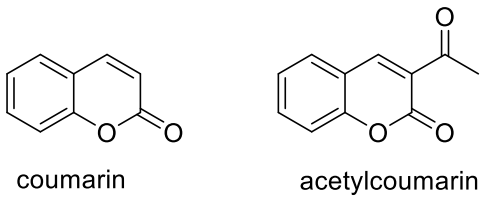


Figure 1.1 Chemical structure of coumarin

Chalcone (α,β-unsaturated ketone) is an outstanding secondary metabolite, a naturally occurring compound found in terrestrial plants. Chalcone is known as 1,3-diphenyl-2-propene-1-one, containing a conjugated double bond which is completely delocalised with the π-electrons in the benzene rings, connected by an α,β-unsaturated enone bridge (Hiba et al., 2021). Chalcone adopts the *cis* or *trans* configuration (Figure 1.2), with the *trans* isomer being more stable and predominant from the standpoint of thermodynamics, which makes it the prevailing configuration between the two forms. The *cis* isomer is unstable due to the steric repulsion between the carbonyl group and the A-ring (Gomes et al., 2017). The presence of a double bond in conjugation with the carbonyl group is responsible for the biological activities of chalcones because the absence of this functional group makes them inactive (Dhaliwal et al., 2022). The α,β-unsaturated ketone moiety serves as a Michael acceptor, enabling chalcones to interact with biological nucleophiles, such as thiol groups in cysteine residues of enzymes or glutathione, leading to downstream biological effects, this reactivity is fundamental to their diverse pharmacological properties, including antimicrobial, anticancer, anti-inflammatory, and antioxidant activities (Gomes et al., 2017). The absence of this crucial functional group, or modifications that disrupt the conjugation, generally leads to a significant reduction or complete loss of activity (Zhuang et al., 2017).

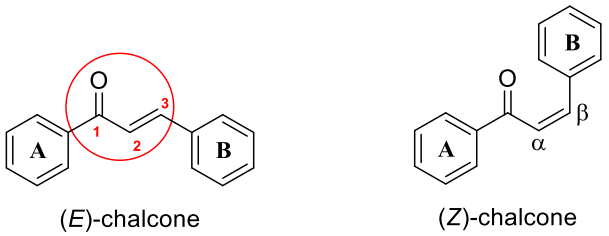


Figure 1.2 The *trans* and *cis* isomers of chalcone

Chalcone functions as a precursor in the biosynthesis of flavonoids and isoflavonoids. Research works on chalcone scaffolds have shown a great deal of interest in the synthetic and biological activities whereby these compounds have shown anti-proliferative and tumour-reducing properties (Basappa et al., 2022). This chalcone scaffold remains attractive due to its abundance in plants. However, since the huge demand for chalcone compounds cannot be met using natural sources, chalcones are synthesised using various routes. The most common method to synthesise chalcone is via the Claisen-Schmidt condensation (Elkanzi et al., 2022).

Kurt et al. (2020) synthesised a series of coumarin-chalcone derivatives with urea moieties, designated 5a-k (e.g., 5a: 1-(3-Methoxyphenyl)-3-(4-(3-oxo-3-(2-oxo-2*H*- chromen-3-yl)prop-1-en-1-yl)phenyl)urea; 5j: 1-(4-Nitrophenyl)-3-(4-(3-oxo-3-(2-oxo-2*H*-chromen- 3-yl)prop-1-en-1-yl)phenyl)urea; 5k: 1-(4-(3-Oxo-3-(2-oxo-2*H*-chromen-3-yl)prop-1-en-1- yl)phenyl)-3-(4(trifluoromethyl) phenyl)urea, through a multi-step process starting with Knoevenagel condensation of salicylaldehyde with ethyl acetoacetate using piperidine at room temperature for 15 minutes to form 3-acetylcoumarin (85% yield), followed by Claisen-Schmidt condensation with p-nitrobenzaldehyde in ethanol with piperidine at 80 °C for 6 hours to yield 3-(3-(4-nitrophenyl)acryloyl)-2*H*-chromen-2-one (3), which was reduced with $SnCl_2 \cdot 2H_2O$ in ethanol at 80°C for 2 hours to produce 3-(3-(4-aminophenyl)acryloyl)-2*H*-chromen-2-one (62% yield), and finally reacted with various isocyanates (e.g., 3-methoxyphenyl

isocyanate, 4-nitrophenyl isocyanate, 4-(trifluoromethyl)phenyl isocyanate) in THF with triethylamine at 70 °C overnight, yielding 5a-k (42-82%), for chalcone carbonyl, confirmed by ^{1}H NMR, ^{13}C NMR and, LC-MS. These compounds were tested for antiproliferative activity against H4IIE (rat hepatoma, IC_{50}: 1.62–19.78 μM), HepG2 (human hepatocellular carcinoma, IC_{50}: 2.326 to 11.427 μM), and CHO (non-cancerous, IC_{50}: 3.824 to 28.664 μM) cell lines using LDH and MTT assays, with 5k (IC_{50}: 1.62 μM, H4IIE) and 5j (IC_{50}: 2.326 μM, HepG2) outperforming sorafenib (IC_{50}: 3.45 μM, H4IIE; 7.522 μM, HepG2), inducing S-phase cell cycle arrest (e.g., 5k: 46.4% S-phase) and apoptosis (e.g., 5k: 59%, 5e: 58%), though 5g showed higher toxicity to CHO cells (IC_{50}: 3.824 μM).

Nitrogen-containing heterocycles are a fundamental class of compounds in medicinal chemistry and are widely found in many pharmaceuticals. Their presence in the molecular structure of drugs enhances their ability to interact with biological targets, often leading to a broad range of pharmacological properties. They have become important in the development of new effective drugs for the treatment of cancer (Hosseinzadeh et al., 2018; Kerru et al., 2020). Molecular modelling and pharmacokinetic studies have shown that the incorporation of the nitrogen-containing heterocyclic scaffold in the molecules can change their flexibility, metabolic profiles, polarity and capacity to form hydrogen bonds (Irannejad, 2018).

Among the five-membered heterocyclic derivatives, pyrazolines have drawn great attention due to their various pharmacologically activities and numerous possibilities of structural diversification (Ardiansah, 2017). Pyrazoline, named by Ludwig Knorr in 1883, has played a significant historical role in medicinal chemistry. In the early years of its discovery, pyrazoline derivatives were used to treat pain, inflammation, and fever (Dash & Karim, 2020). Pyrazoline compounds can be found

in natural products such as vitamins, alkaloids and pigments. Compounds with pyrazoline ring have shown interesting pharmacological activities such as anticancer, antioxidant, antimicrobial, antimalarial and antiviral. These compounds are not only found in nature but can also be synthesised (Matiadis & Sagnou, 2020). Pyrazoline is a five-membered heterocyclic compound having two nitrogen atoms within its ring structure, which contributes to its aromatic properties. This compound and its derivatives exhibit a broad spectrum of biological activities, making them significant in medicinal chemistry (Haider et al., 2022).

Pyrazolines can be classified into three types based on the position of the double bonds and the degree of saturation within the five-membered ring. These types are 1-pyrazoline, 2-pyrazoline, and 3-pyrazoline, as shown in Figure 1.3. Among its derivatives, 2-pyrazoline scaffold is the most frequently studied (Ardiansah, 2017). Compounds with a 2-pyrazoline ring display a broad spectrum of potential pharmacological activities as different methods have been reported for their synthesis (Jadhav et al., 2018).

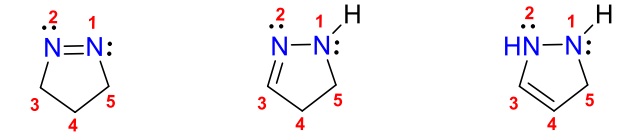


Figure 1.3 Different types of pyrazolines

Amin et al. (2013) synthesised two series of coumarin–pyrazoline hybrids, designated 7a–f, 8a–f, 9a–f, and 10a–f (e.g., 9a: 8-[1-(4-Chlorophenylsulfonyl)-5-(5-methylfuran-2-yl)- 4,5-dihydro-1*H*-pyrazol-3-yl]-7-methoxy-2*H*-chromen-2-one, via Claisen–Schmidt condensation of 7-methoxy-8-acetylcoumarin with various aryl aldehydes (e.g., 5-methylfuran-2-carbaldehyde, 4-methylthiobenzaldehyde) in ethanol

using piperidine at 60-80 °C for 4-6 hours to form chalcone intermediates (7-methoxy-8-(arylacryloyl)-2*H*-chromen-2-ones, 6a-f), followed by cyclisation with substituted phenylsulfonyl hydrazines (e.g., phenylsulfonyl hydrazine, 4-chlorophenylsulfonyl hydrazine, 4-sulfamoylphenyl hydrazine) in refluxing ethanol (80 °C, 8 hours), achieving yields of 50-85%, confirmed by IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry. These compounds were evaluated by the National Cancer Institute (NCI) against 60 cancer cell lines at a single dose (10^{-5} M), with 7d, 8c, and 9c showing significant cytotoxicity against MCF-7 (breast cancer) and HCT-116 (colon cancer); subsequent five-dose assays (0.01–100 μM) revealed 9c as the most potent against HCT-116 (IC_{50}: 0.01 μM, GI_{50}: low micromolar), surpassing doxorubicin (IC_{50}: 0.63 μM), while 7d and 8c exhibited comparable activity; however, 9c showed weak PI3K (p110α/p85α) inhibitory activity, suggesting alternative cytotoxic mechanisms, though compounds 7a, 7b, 7f, and 10a displayed negligible antitumor effects.

Hybrid compounds with a combination of two or more known scaffolds that are covalently linked have been reported to exhibit greater pharmacological potential due to the sum of each scaffold′s potencies. Such compounds have improved pharmacological profile because they can simultaneously target more than one mechanism which can enhance the activity or selectivity and also reduce the adverse drug effects. In this study, hybrid compounds that incorporate coumarin and chalcone scaffolds, as well as coumarin and pyrazoline scaffolds, have been designed and extensively studied for their promising pharmacological properties. The combination of these bioactive scaffolds in a single molecule can lead to enhanced therapeutic efficacy, selectivity, and often a reduction in side effects due to the synergistic effects of the two pharmacologically active moieties.

1.2 Problem Statement

Chemotherapeutic drugs played a critical role in cancer treatment for decades, offering hope for remission or cure to patients suffering from various types of cancer. Despite significant advancements in chemotherapy and cancer treatment, there remains a major challenge: no drugs currently exist that exclusively target only cancer cells without affecting normal, healthy cells. Most chemotherapy agents are non-selective, in which they attack rapidly dividing cells, both cancer cells and healthy cells such as those in the bone marrow, digestive tract, hair follicles, and skin, ultimately inhibiting their division and lead to their destruction. A significant limitation of traditional chemotherapy is its lack of selectivity, the inability to distinguish between normal healthy cells and cancer cells (Kaur et al., 2022). This results in various side effects and organ toxicities, which can significantly reduce the quality of life for cancer patients (Zhong et al., 2021).

Derivatives of coumarin-chalcone and coumarin-pyrazoline, with different substituent which permit interaction with several receptors, have become prominent candidates for the creation of new hybrid compounds. This study aimed to design and synthesise coumarin-chalcone and coumarin-pyrazoline derivatives, followed by *in silico* analysis involving molecular docking, pharmacokinetic (ADME) and drug-likeness predictions, as well as molecular dynamic simulations. The goal was to identify promising candidates for subsequent *in vitro* cytotoxicity testing against MCF-7 breast cancer cell lines.

In addition, the DPPH assay was employed to assess the antioxidant activity of the synthesized compounds. Since oxidative stress is closely linked to cancer progression, evaluating antioxidant capacity provides insight into the potential dual role of these compounds – not only as cytotoxic agents but also as protectors against

oxidative damage in healthy cells. This preliminary screening supports the identification of compounds with both anticancer and antioxidant properties, which were further considered for *in vitro* cytotoxicity testing against MCF-7 breast cancer cell lines.

1.3 Objectives

This research work focuses on four main objectives:

1. To draw the hybrid molecules and perform the preliminary screening via *in silico* study analysis using AutoDock Vina and SwissADME tool.
2. To synthesise new series of coumarin-chalcone derivatives via a Claisen-Schmidt condensation reaction and corresponding coumarin-pyrazoline derivatives through the cyclo-condensation of the chalcones, and to characterise all the synthesised compounds using Fourier Transform Infrared spectroscopy (FT-IR) and Nuclear Magnetic Resonance (NMR) spectroscopy (^{1}H and ^{13}C NMR).
3. To determine the antioxidant activity via DPPH assay and *in vitro* cytotoxicity activity via the MTT assay of the synthesised compounds against MCF-7 breast cancer cell lines.

1.4 Scope of Study

This research will involve the synthesis, computational assessment, and cytotoxicity evaluation of new coumarin-based hybrid compounds containing chalcone and pyrazoline scaffolds, with the aim of identifying potential therapeutic agents against breast cancer. 62 compounds will be sketched using ChemDraw 20.0 and subsequently subjected to *in silico* evaluation through molecular docking targeting the estrogen receptor alpha (PDB ID: 3ERT), along with pharmacokinetic and drug-

likeness assessments using the SwissADME web tool. Compounds exhibiting favourable binding affinities and promising pharmacokinetic profiles from the virtual screening will be selected for chemical synthesis.

The synthesised compounds will be characterised using FTIR, ^{1}H, ^{13}C, and 2D NMR spectroscopy to confirm their molecular structures. An initial antioxidant screening will be conducted using the DPPH assay to identify the most biologically active candidates. These selected compounds will then be evaluated for their *in vitro* cytotoxic effects against MCF-7 cell lines using the MTT assay. Additionally, L-929 normal fibroblast cell lines will be used to assess the selectivity of the compounds, thereby providing insights into their therapeutic potential as anticancer agents.

CHAPTER 2

LITERATURE REVIEW

2.1 Molecular modelling

Molecular modelling is a powerful computational technique that enables researchers to study the structure, dynamics, and interactions of molecules at the atomic level. This technique has become indispensable in understanding the behaviour of molecules and designing new compounds with desired properties, such as potential drug candidates or materials with specific functions (Peluso & Chankvetadze, 2024).

Molecular modeling technologies have created automated platforms for computer-aided drug design (CADD), offering a cost-effective and efficient method for identifying and optimising drug candidates. By integrating computational methods with experimental research, CADD enables researchers to predict the biological activity of potential drug compounds based on their molecular structure. This accelerates the discovery of potential therapeutic compounds, focusing on the most promising drug candidates in the early stage of development process (Kaur & Khatik, 2021; Niazi & Mariam, 2024).

In CADD, Structure-Based Drug Design (SBDD) and Ligand-Based Drug Design (LBDD) are two commonly used techniques that guide the development of new therapeutic drugs, as shown in Figure 2.1. In SBDD, the target(s) protein is known, and the molecule is designed to best fit to the active site of the protein to give the interaction and also the biological effects of the interactions. Meanwhile, in LBDD, the protein structure is not known, and it depends on the chemical structure of the known active compound (ligand) that have shown activity against the target. Pharmacophores are created to model the key features responsible for the activity (van Montfort & Workman, 2017; Adelusi et al., 2022). Two primary SBDD techniques are molecular

docking and molecular dynamics. In recent years, the demand for molecular docking approaches in drug discovery and development has increased unexpectedly.

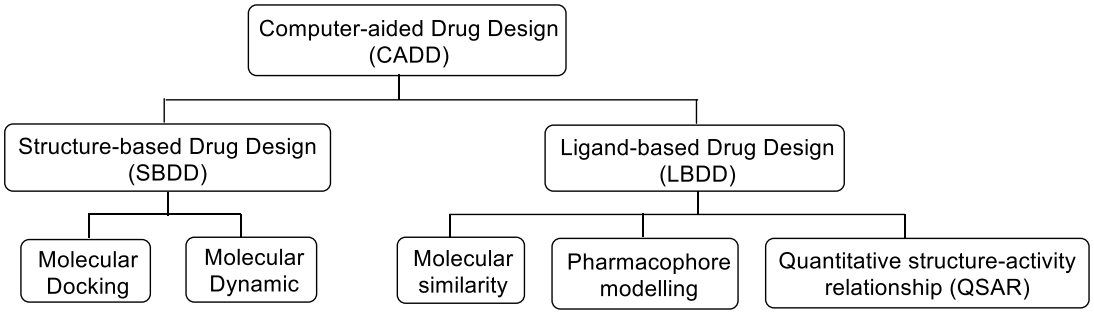


Figure 2.1 Structure of Computer-aided Drug Design (CADD)

2.1.1 Molecular Docking

Molecular docking is a commonly used technique in drug discovery and has been applied to various target proteins, including enzymes, receptors, and ion channels. It can be used to identify new lead compounds, optimise existing compounds, and understand the binding mechanisms of ligands to target proteins (Wang & Zhu, 2016). Numerous docking programs such as DOCK 5 (Moustakas et al., 2006), AutoDock (Morris et al., 2009), GLIDE (Friesner et al., 2006), AutoDock Vina (Trott & Olson, 2010), and GOLD (Verdonk et al., 2003) are available. However, molecular docking has some limitations, such as the accuracy of the scoring functions and the ability to account for protein flexibility and solvation effects. Therefore, molecular docking is often combined with other computational and experimental techniques such as GOLD and CSCORE to improve its accuracy and reliability (Saikia & Bordoloi, 2019).

Molecular docking techniques can predict how small molecules or ligands will conform when they enter the protein receptor′s active site (Muchtaridi et al., 2017). The interaction forms in a ligand-protein complex predict the biochemical phenomenon and the effectiveness of the ligand-protein binding is determined by the

virtual binding energy values. Molecular docking involves two key steps which are sampling and scoring. In the sampling step, various conformations of the ligand are generated and docked into the binding site of the receptor using different sampling algorithms. This is to explore the conformational space of the ligand and to identify the most favourable binding mode. In the scoring step, the generated conformations are evaluated using different scoring functions to estimate their binding affinity and eventually identify the most promising ligands (Agu et al., 2023).

2.1.2 Molecular Dynamic

Molecular dynamics (MD) simulations are a powerful computational technique used to model and study the physical movements of atoms and molecules over time. In drug discovery and other fields of molecular research, MD simulations provide insight into the dynamic behaviour of biological systems and mechanisms underlying biological functions, such as proteins, DNA, lipids, and small molecules (like drugs), under various conditions (Salo-Ahen et al., 2021). MD simulations can reveal how small molecules (drugs) bind to proteins in a more realistic, dynamic environment compared to static molecular docking. It complements other techniques such as molecular docking and offers more accurate predictions of how drugs interact with their targets over time. MD can also simulate the process of ligand association and dissociation to understand binding kinetics and thermodynamics. It allows for the exploration of induced fit effects, where the protein′s binding site adapts to the ligand (Badar et al., 2020).

2.2 *In silico* ADME properties

SwissADME is a web tool that provides a comprehensive evaluation of drug-likeness and pharmacokinetic parameters of small molecules (Daina et al., 2017). Developed by the Swiss Institute of Bioinformatics (SIB), it allows researchers to assess

critical factors that influence the behaviour of drug candidates in the body, such as their absorption, distribution, metabolism, excretion, and toxicity (ADMET). SwissADME is an indispensable tool for early-stage drug discovery, providing essential insights into the physicochemical and pharmacokinetic, and drug-likeness properties of small molecules, guiding lead selection and optimisation (Daina & Zoete, 2019). The *in silico* ADME analysis helps to eliminate compounds and select promising drug candidates with favourable ADME properties early in the drug discovery process, thereby reducing the time and cost associated with experimental testing. The ADME (Absorption, Distribution, Metabolism, and Excretion) properties of effective drug molecules justify the drug compound′s ability to process inside a living organism (Dar et al., 2019).

2.3 Coumarin-chalcone compounds

The coumarin nucleus and the α,β-unsaturated ketone system of chalcones provide structural diversity to these hybrids. Coumarin-chalcone hybrids represent a fascinating class of compounds that combine two well-known bioactive scaffolds, coumarin and chalcone, two naturally occurring chemical scaffolds, into a single molecular framework that exhibit a broad spectrum of biological activities, These hybrids are created by chemically linking the coumarin and chalcone moieties, aiming to enhance to enhance therapeutic properties through synergistic effects, which makes them promising candidates for drug development (Wei et al., 2016). Coumarins and chalcone scaffolds, both exhibit anticancer activity by inducing apoptosis, inhibiting cell proliferation, and interfering with cancer cell signalling pathways. These compounds also have free radical scavenging activities, protecting cells from oxidative stress and reduce oxidative damage in cells. Chemically merged structures of coumarin and chalcone aims to enhance bioactivity and optimise pharmacokinetic properties,

allowing the hybrid to exert stronger or more diverse biological effects than either moiety alone (Koley et al., 2024).

Modifications in the position and nature of the substituents on either the coumarin or chalcone moieties can lead to significant changes in their biological activity, offering flexibility in designing molecules with target-specific activities. The hybridisation can lead to an improvement in pharmacological potency compared to individual coumarins or chalcones (Akkol et al., 2020). Coumarin-chalcone hybrids represent a promising class of multifunctional compounds that can interact with multiple molecular targets, making them promising agents in drug discovery. Hybridisation helps to improve solubility, stability, and bioavailability, which are key factors in drug development while structural modifications and optimisation enhance the pharmacological profile of these hybrids, leading to the development of new compounds (Das et al., 2022).

2.3.1 Synthesis of chalcone compounds

Although coumarin-chalcone hybrids are easily synthesised compounds, a growing number of new techniques and procedures have recently been reported because of their interesting biological activities. The development of various green catalysts or reaction conditions have also led to newer synthetic routes. There are various methods to synthesise chalcone compounds, with the Claisen-Schmidt condensation being the most common. However, modifications and alternative approaches can also be used depending on the substrates or desired functional groups.

2.3.1(a) Claisen-Schmidt condensation

The Claisen−Schmidt reaction is a reaction which involves condensing a benzaldehyde and a methyl ketone in the presence of either strong base or acid catalysts as shown in Figure 2.1. In the case of base catalysis, the chalcone is generated from the

aldol product via dehydration in an enolate mechanism, while in the case of acid catalysis, it is generated via an enol mechanism (Zhuang et al., 2017).

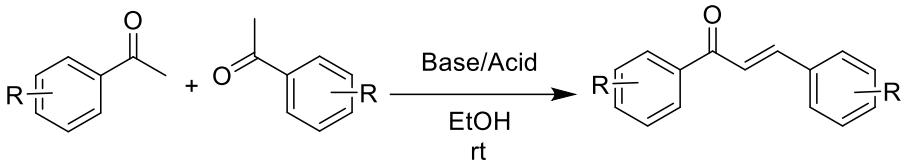


Figure 2.2 Synthesis of chalcone via Claisen-Schmidt condensation

Solvent-free Claisen-Schmidt condensation has been introduced to minimise the use of solvents and to reduce the environmental impacts. This condensation involves the reaction of two carbonyl compounds (aldehydes or ketones) in the absence of any solvent. Figure 2.3 shows the reaction of aryl aldehyde derivatives and acetophenone derivatives that was carried out over a zinc oxide supported with a metal oxide catalyst under solvent-free conditions to produce a chalcone compound. The reaction gave 81% yield with good purity. This method is considered to be environmentally friendly and practical, making it a safe alternative to the other traditional methods (Saravanamurugan et al., 2005).

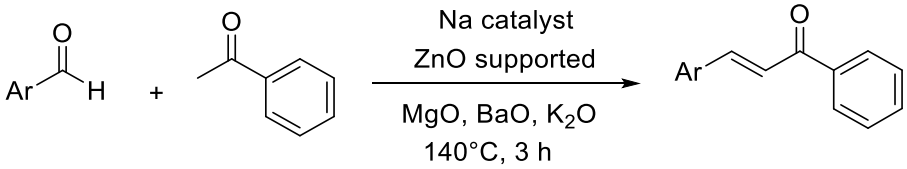


Figure 2.3 Solvent-free Claisen-Schmidt condensation

2.3.1(b) Suzuki-Miyaura Coupling Reaction

Suzuki-Miyaura coupling is an interesting metal-catalysed cross-coupling reaction, in which chalcones were formed from two chemical fragments via the formation of a C-C bond (Figure 2.4). A series of chalcones were synthesised via the

Suzuki-Miyaura coupling of styryl boronic acid and various benzoyl chlorides resulted in very high product yields at 80% (Haddach & McCarthy, 1999).

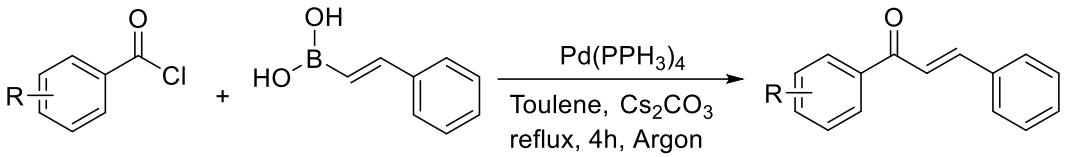


Figure 2.4 Synthesis of chalcone by Suzuki-Miyaura coupling

2.3.1(c) Heck Coupling Reaction

This approach involves new strategies such as cross-coupling reactions using transition metal catalysts. Such syntheses have managed to form several series of new chalcones for pharmaceutical applications (Guo et al., 2015; Zhuang et al., 2017; Díaz-Sánchez et al., 2019). The metal-catalysed Heck coupling reaction is an efficient procedure to form a good yield of chalcones through the coupling of aryl vinyl ketones aryl with aryl iodides or aryl boronic acids under catalytic conditions (Hird et al., 1993). The most common metal catalyst used is Pd(OAc)2, as shown in Figure 2.5. Chalcones have also been prepared via a carbonylative vinylation of aryl halides with styrene in the presence of carbon monoxide using a palladium catalyst (Guo et al., 2015).

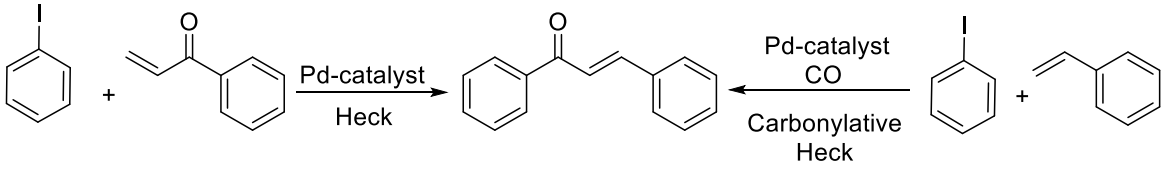


Figure 2.5 Synthesis of chalcone via metal-catalysed Heck coupling reaction

2.3.1(d) Sonogashira Coupling reactions

Sonogashira coupling is a reaction of terminal alkynes with aryl halides in the presence of a palladium catalyst combined with a co-catalytic amount of CuI in a boiling

mixture of trimethylamine and THF, under inert condition for 16-24 hours (Figure 2.6) (Müller et al., 2000). In this Sonogashira Coupling reaction, several desired chalcones were synthesised in moderate to excellent yields. However, Sonogashira coupling has some limitations, such as long reaction times, using an excess base and electron-deficient aryl halides. To overcome these shortcomings, Schramm & Mueller (2006) have proposed a microwave-assisted coupling isomerisation reaction for the synthesis of chalcones, which was completed in less than half-hour.

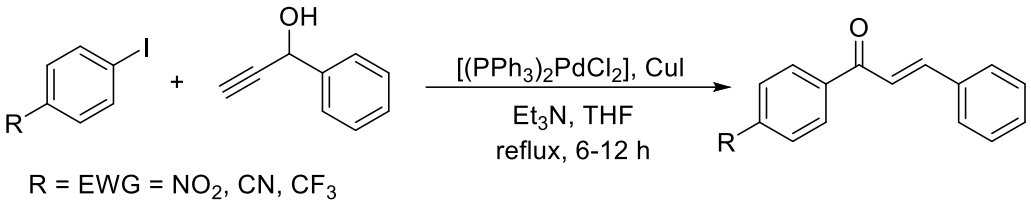


Figure 2.6 Synthesis of chalcones using Sonogashira coupling reaction

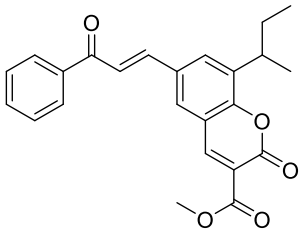
2.3.2 Anticancer activity of coumarin-chalcone compounds

Chemotherapy has long been the most widely used treatment for illnesses, particularly when it comes to eliminating malignant cells. One important area of research is still the chemical modification of potential lead molecules to create the new and effective medications (Wei & Zhang, 2016). Coumarin-chalcone compounds have been found to exhibit significant anticancer activity that may prevent the growth and spread of cancer cells (Leitao, 2020). The IC_{50} is a half-maximal inhibitory concentration, widely used as an informative measure of a drug′s efficacy. It indicates how much drug is needed to inhibit a biological process by half. The approach to determine the IC_{50} of a compound are based on the assays that uses the cell system (Aykul & Martinez-Hackert, 2016).

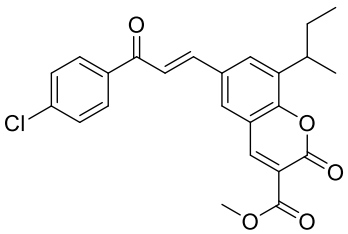
Inspired by the reported antiproliferative activity of some naturally occurring chalcones, Sasidhara and co-workers (2010) reported two series of novel hybrid

compounds containing coumarin and chalcone scaffolds. These compounds were screened *in vitro* for their growth-inhibitory effect against the MCF-7 breast cancer cells. The cytotoxicity evaluation found that among all the compounds, S-1, S-2 and S-3 were active with the IC_{50} values of 11.41 μM, 10.80 μM and 11.07 μM, respectively.

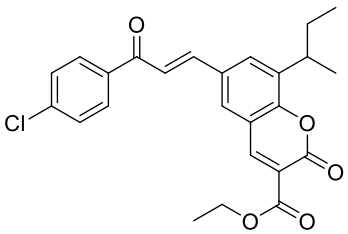
The structure-activity relationship (SAR) investigation revealed that substituents on the coumarin ring influence their anticancer activity whereby the other substituents showed considerable potency.



S-1: IC_{50} = 11.41 μM

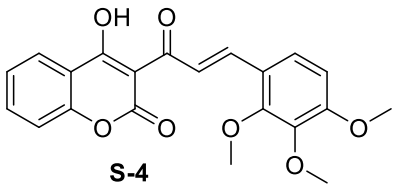


S-2: IC_{50} = 10.80 μM



S-3: IC_{50} = 11.07 μM

Patel et al. (2011) have reported the antiproliferative activity of a series of hybrid coumarin-chalcones. Among these compounds, S-4, with three methoxy substitutions on the phenyl ring, has shown good activity against human breast cancer cells, MCF-7 (GI_{50} = 25.86 ± 0.25 μM), comparable to the reference drug, cisplatin (GI_{50} = 23.65 ± 0.23 μM). The GI_{50} refers to the concentration of a drug that reduces total cell growth by 50%.

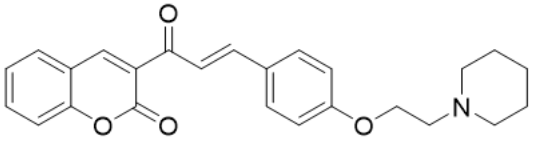


S-4

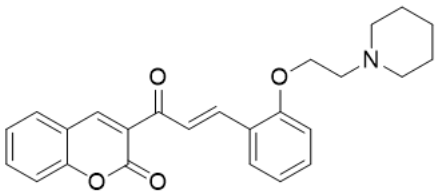
GI_{50} = 25.86 ± 0.25 μM

Mokale et al. (2017) has reported the synthesis and characterisation of nine coumarin-chalcone series namely 3-(3′-(substituted phenyl)acryloyl)-2H-chromen-2-one derivatives. All compounds were examined for their anticancer activities against

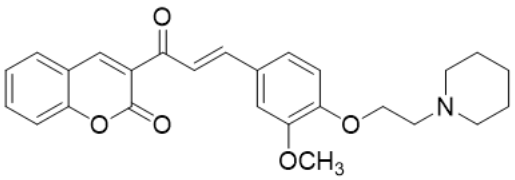
the MCF-7 cell lines. The results showed that three compounds (S-5, S-6 and S-7) were active against the MCF-7. Compound S-5 showedthe strongest anti-proliferative action (GI_{50} = <10 mg/mL), followed by S-6 in which the substituent is at different position (GI_{50} = 18.9 mg/mL) while the addition of methoxy at phenyl of compound S-7 (GI_{50} = 32.5 mg/mL) reduced the activity. Compound S-5 was found to have similar effect with the reference drug, Adriamycin (GI_{50} = <10 mg/mL) and Tamoxifen (GI_{50} = <10 mg/mL).



S-5 (GI_{50} = < 10 mg/mL)

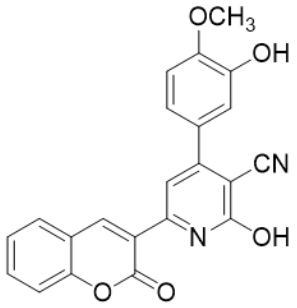


S-6 (GI_{50} = 18.9 mg/mL)

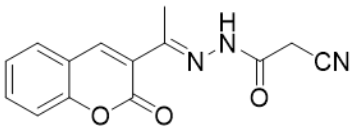


S-7 (GI_{50} = 32.5 mg/mL)

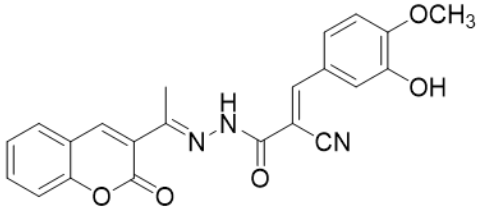
Several coumarin-chalcone compounds with good cytotoxicity activity have been reported by Abdel-Latif and co-workers (2018). These compounds, S-8 to S-11, showed promising IC_{50} values, comparable to the reference drug used, Doxorubicin (IC_{50} = 4.17 ± 2.6 μM).



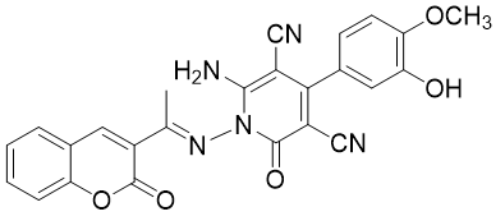
S-8: IC_{50} = 11.26 ± 1.1 μM



S-9: IC_{50} = 6.34 ± 0.5 μM

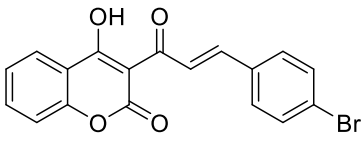


S-10: IC_{50} =8.92 ± 0.7 μM



S-11: IC_{50} =17.01 ± 1.4 μM

Patel et al (2021) reported a series of coumarin-chalcone compounds with the ability to be effective anti-proliferative agents. The positions of substituents on the phenyl moiety have been highlighted. Among all the synthesised compounds, S-12 showed the most anti-proliferative effect against breast cancer cells (MCF-7) with the GI_{50} value of 25.86 ± 0.25 μM, comparable to the reference drug, Cisplatin (GI_{50} = 25.77 ± 0.38 μM).



S-12

GI_{50} = 25.86 ± 0.25 μM

Chan and coworkers (2022) have reported a water-soluble chemo-sensor, **S-13**, consisting of a chalcone-coumarin hybrid was reported by Chan and co-workers (2022). **S-13** showed excellent selectivity and sensitivity towards Al^{3+} ions, and exhibited