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Synthesis, Characterization, Cytotoxicity Study and Docking Studies of New Fused-pyrazoline Derivatives derived from Bis-Chalcones against Breast Cancer cells

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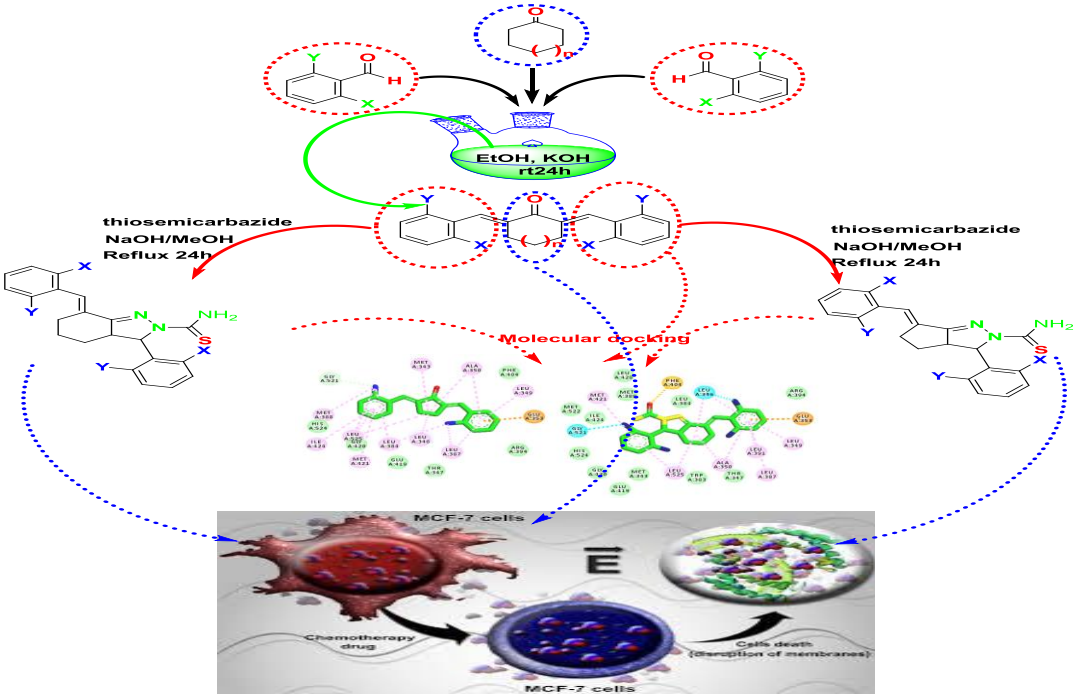
Abstract

Breast cancer is one of the prevalent diseases that kill millions of women worldwide. The development of resistance and side effects of chemotherapy drugs is a common obstacle in the treatment of breast cancer. Recently, the focus of drug discovery has increased toward a valuable structure known as chalcones due to their extensive bioactivity in cancer treatment. In this study, 6 chalcones and 4 fused-pyrazoline derivatives have been synthesized **(1-6)** using the Claisen-Schmidt condensation technique for cyclopentanone and cyclohexanone with different aromatic aldehydes at room temperature. Chalcones **2-3** and **5-6** were used in cyclo-condensation reaction with thiosemicarbazide to form new fused pyrazoline derivatives. All the synthesized chalcones and fused pyrazoline were characterized using ATR-FTIR, H-NMR and ^{13}C-NMR (1D). The cytotoxic activity of these new chalcone compounds was investigated against breast cancer cell lines (MCF-7) and normal breast cell lines (MCF-10A). The results showed that compounds **1** and **10** exhibited significant antiproliferative effects against MCF-7 with IC_{50} values of 8 µM and 8.5 µM when exposed for 48 hours compared with the reference anticancer drug, tamoxifen. Molecular docking analysis showed that compounds **1** and **10** entered the pocket of ERα and interaction with the amino acids in high affinity of binding as tamoxifen.

Keywords: fused-pyrazoline, cyclopentanone, cyclohexanone, Docking Studies, cytotoxicity.

Graphical Abstract



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1. Introduction

Cancer is a current major health problem worldwide in which the body cells proliferate uncontrollably to form a tumor [1]. According to the World Health Organization (WHO), cancer is the second leading cause of death globally after cardiovascular disease with breast cancer as the most prevalent cancer, followed by lung and colorectal cancers, which lead to approximately 9.6 million deaths in 2018. Thus, there is an urgent need to search for an effective approach to fight this cancer [2, 3]. Although treatment with chemotherapeutic drugs inhibits the growth of cancer cells, usually they are not selective and also kill normal cells, causing the patients to suffer from different side effects [4]. Over the last 30 years, Estrogen receptors (ERα and ERβ) are vital regulators of breast cancer development [5]. ERα-positive breast tumors represent 56% of all breast cancer cases, known as hormonal-dependent type breast cancer [5]. Thus anti-estrogen, tamoxifen, is considered the primary treatment for hormonal breast cancer. Tamoxifen blocks the estrogenic signal and binds to the estrogen receptors, thereby modifying its activity and inhibiting cancer cell growth. Tamoxifen and its active metabolite 4-hydroxytamoxifen (4-OHT) have cytotoxic activity against MCF-7 breast cancer cells. However, the efficacy of tamoxifen is limited by the presence of potential drug resistance [6- 8].

Chalcones or 1,3-diphenyl-2-propen-1-ones have been proven to display a broad spectrum of pharmacological activities, including anticancer [9,10]. They are made up of two benzene rings with a diverse array of substituents which are interconnected by a highly electrophilic three-carbon α,β-unsaturated carbonyl system, as shown in Figure 1 [11,12].

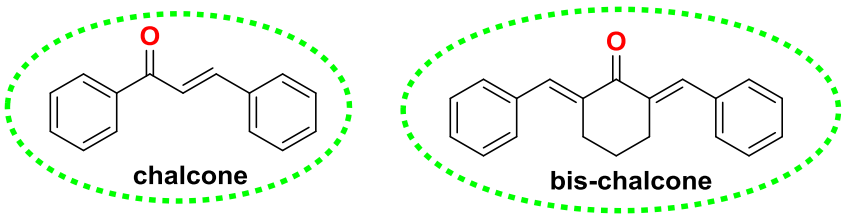


Figure 1: The general structure of chalcone and bis-chalcone.

Curcumin has been proven to possess diverse biological activities, primarily anticancer, towards a variety of human cancer cell lines, including breast cancer cells [13]. Most importantly, curcumin has been reported to selectively kill tumor cells but not the normal cells [14, 15]. However, due to its active methylene moiety, the clinical efficacy of curcumin is limited by its low stability at pH above 6.5 [16]. Therefore, curcumin analogs have been synthesized by modifying the methylene group into either cyclopentanone or cyclohexanone to improve their stability in biological medium as shown in Figure 2 [15-16].

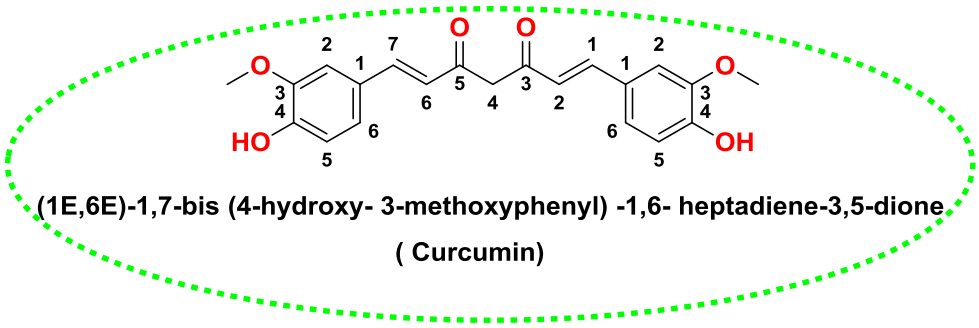


Figure 2: The general structure of Curcumin.

Chalcones are key precursors in the synthesis of many biologically important heterocycles such as pyrazolines [17]. Pyrazoline is a lot of interest from researchers due to its various biological activities as shown in Figure 3 [18]. Previous research works proved that pyrazolines with carbothioamide group attached to its N^1 position showed good anticancer activity against breast cancer cell lines [19]. Besides, halogen substituents on the aromatic ring attached to the pyrazoline scaffold help enhance the cytotoxicity of pyrazoline compounds [17]. However, the reports on the anticancer activity of fused-pyrazoline derivatives from bis-chalcones are scarce compared to the pyrazoline derivatives formed from mono-chalcones [20]. Fused-pyrazoline derivatives have only being studied on other biological properties such as antimicrobial, antibacterial, anti-fungal, and anti-inflammatory by previous researchers [19-20].

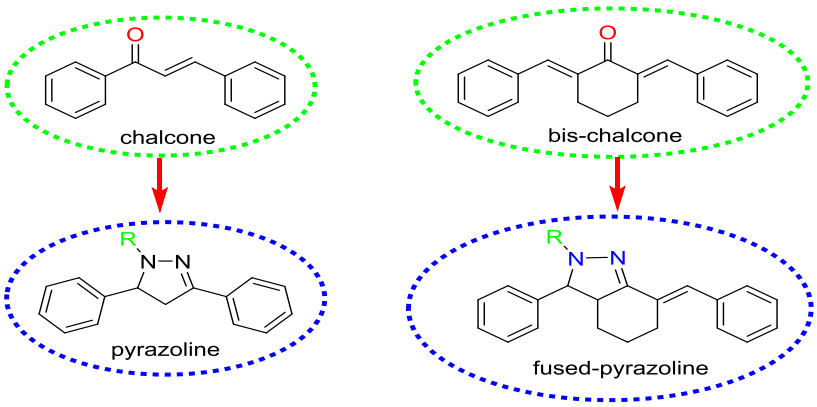


Figure 3: Different types of pyrazolines

As part of an effort to discover safer and more efficient anticancer agents, we report here the synthesis of a series of new fused-pyrazoline compounds derived from bis-chalcones and their cytotoxicity against a breast cancer cell lines (MCF-7).

2. Materials and methods

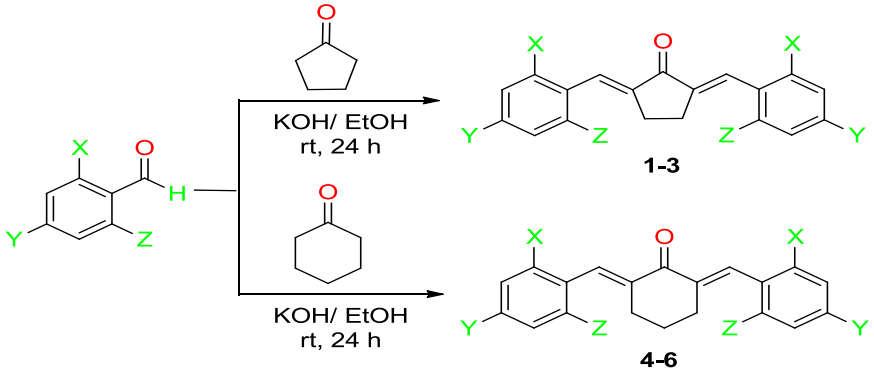
2.1 Experimental section

2.1.1 Synthesis methods

All the chemicals and solvents were used without further purification. IR spectra were obtained using a Perkin Elmer Frontier FT-IR Spectrometer with Perkin Elmer Universal ATR Sampling Accessory. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 500 MHz Ultrashield™ spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) with deuterated chloroform ($CDCl_3$) as the solvent.

2.1.2 General procedure of Bis-chalcones, 1-6

A series of chalcones were prepared *via* "Claisen -Schmidt" condensation (Scheme 1) between a series of substituted benzaldehydes with cyclopentanones (**1-3**) and cyclohexanone (**4-6**). Cyclopentanone (1.00 g, 0.012 moles) and benzaldehyde (2.53 g, 0.024 moles) were mixed with ethanol (50.0 mL) in a 250 mL round-bottom flask. Potassium hydroxide pellets (2.00 g, 0.036 moles) were added and the mixture was stirred at room temperature for 24 hours. The reaction progress was monitored using TLC. Upon completion, the precipitate formed was filtered and dried. Recrystallization from methanol gave the expected compound.



Compounds	X	Y	Z
1	Cl	H	H
2	Cl	H	Cl
3	F	H	Cl
4	Cl	H	H
5	Cl	H	Cl
6	F	H	Cl

Scheme 1: Synthesis of bis-chalcones **1-6**

2,5-Bis((*E*)-2-chlorobenzylidene]cyclopentan-1-one, 1

Yellow solid; yield: 3.55 g (90.6 %). IR (ν, cm^{-1}): 3071 (alkene Csp^2-H stretch), 3029 (aromatic Csp^2-H stretch), 2975 (alkyl Csp^3-H stretch), 1687 (conjugated C=O stretch), 1599 (aromatic C=C stretch), 751 (C-Cl stretch). ^{1}H-NMR (500 MHz, $CDCl_3$) δ, ppm: 3.02 (s, 4H, CH_2), 7.31-7.35 (m, 4H, H-Ar), 7.48 (dd, J = 7.0 Hz, 2.6 Hz, 2H, H-Ar), 7.57 (dd, J = 7.0 Hz, 2.6 Hz, 2H, H-Ar), 7.95 (s, 2H, CH).

^{13}C-NMR (125 MHz, $CDCl_3$) δ, ppm: 26.6,126.6, 130.1, 130.1, 130.1, 130.2, 133.9, 136.1, 139.3, and 195.6.

2,5-Bis((*E*)-2,6-dichlorobenzylidene)cyclopentan-1-one, 2

Yellow solid; yield: 4.45 g (93.9 %). IR (ν, cm^{-1}): 3067 (alkene Csp^2-H stretch), 3021 (aromatic Csp^2-H stretch), 2971 (alkyl Csp^3-H stretch), 1704 (conjugated C=O stretch), 1631 (aromatic C=C stretch), 767 (C-Cl stretch). ^{1}H-NMR (500 MHz, $CDCl_3$) δ, ppm: 2.63 (s, 4H, CH_2), 7.25 (t, J = 8.0 Hz, 2H, H-Ar), 7.38 (d, J = 8.0 Hz, 4H, H-Ar), 7.53 (s, 2H, CH). ^{13}C-NMR (125 MHz, $CDCl_3$) δ, ppm: 31.0, 128.1, 128.8, 129.7, 133.8, 134.5, 143.1 and 194.2.

2,5-Bis((*E*)-2-chloro-6-fluorobenzylidene)cyclopentan-1-one, 3

Yellow solid; yield: 3.23 g (74.4 %). IR (ν, cm^{-1}): 3091 (alkene Csp^2-H stretch), 3025 (aromatic Csp^2-H stretch), 2938 (alkyl Csp^3-H stretch), 1704 (conjugated C=O stretch), 1602 (aromatic C=C stretch), 1192 (C-F stretch), 768 (C-Cl stretch). ^{1}H-NMR (500 MHz, $CDCl_3$) δ, ppm: 2.70 (s, 4H, CH_2), 7.08 (t, J = 8.5 Hz, 2H, H-Ar), 7.27-7.34 (m, 4H, H-Ar), 7.55 (s, 2H,CH). ^{13}C-NMR (125 MHz, $CDCl_3$) δ, ppm: 26.2, 114.5, 123.1, 124.8, 125.5, 130.4, 135.7, 143.4, 160.1 and 194.4.

2,6-Bis((*E*)-2-chlorobenzylidene)cyclohexan-1-one, 4

Yellow solid; yield: 3.09 g (88.3 %). IR (ν, cm^{-1}): 3064 (alkene Csp^2-H stretch), 3025 (aromatic Csp^2-H stretch), 2971 (alkyl Csp^3-H stretch), 1663 (conjugated C=O stretch), 1601 (aromatic C=C stretch), 733 (C-Cl stretch). ^{1}H-NMR (500 MHz, $CDCl_3$) δ, ppm: 1.76-1.81 (m, 2H, CH_2), 2.80 (t, J = 5.3 Hz, 4H, CH_2), 7.29-7.31 (m, 4H, H-Ar), 7.36 (dd, J = 5.5, 4.0 Hz, 2H, H-Ar), 7.46 (dd, J = 5.5 Hz, 4.0 Hz, 2H, H-Ar), 7.93 (s, 2H, CH). ^{13}C-NMR (125 MHz, $CDCl_3$) δ, ppm: 23.2, 28.4, 126.3, 129.6, 129.8, 130.5, 134.1, 134.4, 135.0, 137.8 and 189.8.

2,6-Bis((*E*)-2,6-dichlorobenzylidene) cyclohexan-1-one, 5

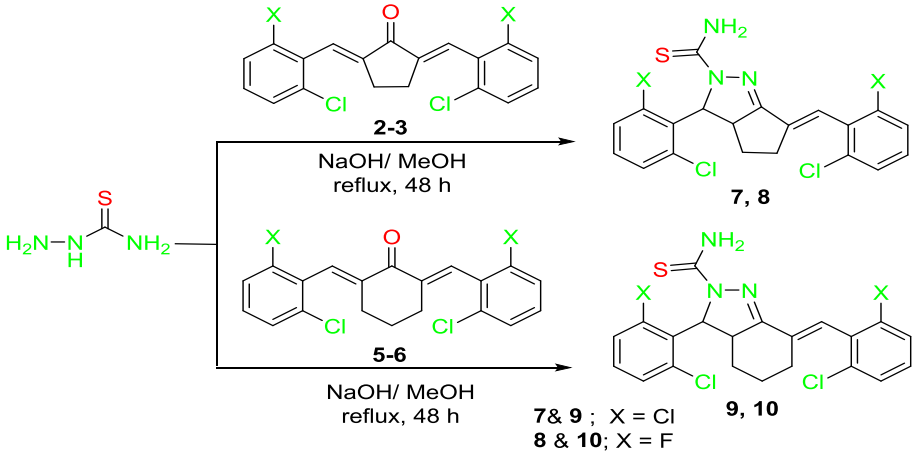
Yellow solid; yield: 4.46 g (94.7 %). IR (ν, cm^{-1}): 3075 (alkene Csp^2-H stretch), 2945 (alkyl Csp^3-H stretch), 1677 (conjugated C=O stretch), 1604 (aromatic C=C stretch), 776 (C-Cl stretch), ^{1}H-NMR (500 MHz, $CDCl_3$) δ, ppm: 1.73-1.78 (m, 2H, CH_2), 2.48 (t, J = 5.0 Hz, 4H, CH_2), 7.23 (t, J = 8.0 Hz, 2H, H-Ar), 7.37 (d, J = 8.0 Hz, 4H, H-Ar), 7.62 (s, 2H, H-Ar). ^{13}C-NMR (125 MHz, $CDCl_3$) δ, ppm: 22.0, 28.2, 128.0, 129.4, 132.3, 134.2, 134.5, 140.2 and 188.1.

2,6-Bis((*E*)-2-chloro-6-fluorobenzylidene)cyclohexan-1-one, 6

Yellow solid; yield: 3.10 g (64.7 %). IR (ν, cm^{-1}): 3067 (alkene Csp^2-H stretch), 3029 (aromatic Csp^2-H stretch), 2971 (alkyl Csp^3-H stretch), 1663 (conjugated C=O stretch), 1601 (aromatic C=C stretch), 735 (C-Cl stretch), ^{1}H-NMR (500 MHz, $CDCl_3$) δ, ppm: 1.73-1.78 (m, 2H, CH_2), 2.57 (t, J = 6.0 Hz, 4H, CH_2), 7.06 (t, J = 8.0 Hz, 2H, H-Ar), 7.26-7.32 (m, 4H, H-Ar), 7.60 (s, 2H, CH). ^{13}C-NMR (125 MHz, $CDCl_3$) δ, ppm: 22.4, 28.7, 114.3, 123.5, 125.3, 128.2, 130.0, 135.3, 140.7, 159.8, and 188.3.

2.1.3 Synthesis of fused-pyrazoline derivatives, 7-10

New chalcone compounds, **2, 3, 5** and **6** were used in cyclo-condensation reaction (Scheme 2) with thiosemicarbazide to form the new fused-pyrazoline compounds**, 7, 8, 9** and **10**. Compound **2** was used as a representative in this procedure. Chalcone **2** (1.00 g, 2.51 mmol) and thiosemicarbazide (0.34 g, 3.77 mmol) were mixed with methanol (30 mL) in a 250 mL round-bottom flask. Sodium hydroxide pellets (0.20 g, 5.02 mmol) was added and the reaction mixture was refluxed for 48 hours. The reaction progress was monitored using TLC. Upon completion, distilled water was added and the precipitate formed was filtered and dried. Recrystallization from the pet-ether gave a yellow powder.



Scheme 2: Synthesis of fused-pyrazoline derivatives

(*E*)-6-(2,6-dichlorobenzylidene)-3-(2,6-dichlorophenyl)-3a,4,5,6-tetrahydrocyclopenta[c] pyrazole-2(3*H*)-carbothioamide, 7

Yellow solid; yield: 0.77 g (65.0 %). IR (ν, cm^{-1}): 3250, 3426 (N-H stretch), 3148 (alkene Csp^2-H stretch), 2955 (alkyl Csp^3-H stretch), 1582 (C=N stretch), 1497 (aromatic C=C stretch), 1284 (C-N stretch), 1087 (C=S stretch), 774 (C-Cl stretch). ^{1}H-NMR (500 MHz, $CDCl_3$) δ, ppm: 1.77-1.81 (m, 2H, CH_2), 2.54-2.64 (m, 2H, CH_2), 4.02-4.09 (m, 1H, CH), 6.12 (d, J = 9.9 Hz, 1H, CH), 7.17 (s, 1H, NH_2), 7.34-7.39 (m, 4H, H-Ar). ^{13}C-NMR (125 MHz, $CDCl_3$) δ, ppm: 23.9, 28.0, 33.5, 58.0, 122.7, 128.1, 128.6, 129.5, 129.9, 133.3, 134.0, 134.4, 134.5, 134.8, 167.6 and 177.8. CHN Elemental analysis for **$C_{20}H_{15}Cl_4N3S$**: Calculated: C, 50.98; H, 3.21; N, 8.92; Found: C, 50.53; H, 3.37; N; 8.86.

(*E*)-6-(2,6-dichlorobenzylidene)-3-(2,6-dichlorophenyl)-3a,4,5,6-tetrahydrocyclopenta[c] pyrazole-2(3*H*)-carbothioamide, 8

Yellow solid; yield: 0.76 g (63.6 %). Yellow solid. IR (ν, cm^{-1}): 3250, 3431 (N-H stretch), 3145 (alkene Csp^2-H stretch), 2956 (alkyl Csp^3-H stretch), 1575 (C=N stretch), 1445 (aromatic C=C stretch), 1278 (C-N stretch), 1060 (C=S stretch), 774 (C-Cl stretch). ^{1}H-NMR (500 MHz, $CDCl_3$) δ, ppm: 1.88-1.96 (m, 2H, CH_2), 2.54 (t, J = 7.5 Hz, 2H, CH_2), 3.98-4.04 (m, 1H, CH), 6.54 (d, J=11.8 Hz, 1H, CH), 7.15 (s, 1H, CH), 7.24-7.29 (m, 6H, H-Ar), 8.25 (s, 1 H, NH), 8.69 (s, 1H, NH). ^{13}C-NMR (125 MHz, $CDCl_3$) δ, ppm: 22.0, 28.2, 31.2, 54.3, 119.3, 122.3, 123.2, 123.9, 125.3, 125.4, 125.7, 126.2, 129.9, 131.1, 134.2, 134.6, 135.1, 142.9, 168.0 and 178.8. CHN Elemental analysis for **$C_{20}H_{15}Cl_2F_2N_3S$**: Calculated: C, 54.80; H, 3.45; N, 9.59; Found: C, 54.33; H, 3.37; N; 9.61.

(*E*)-7-(2,6-dichlorobenzylidene)-3-(2,6-dichlorophenyl)-3,3a,4,5,6,7-hexahydro-2*H*-indazole-2-carbothioamide, 9

White solid; yield: 0.83 g (70.6 %). IR (ν, cm^{-1}): 3253, 3430 (N-H stretch), 3150 (alkene Csp^2-H stretch), 3062 (aromatic Csp^2-H stretch), 2934 (alkyl Csp^3-H stretch), 1592 (C=N stretch), 1561 (aromatic C=C stretch), 1229 (C-N stretch), 1109 (C=S stretch), 779 (C-Cl stretch). ^{1}H-NMR (500 MHz, $CDCl_3$) δ, ppm: 1.48-1.68 (m, 2H, CH_2), 1.78-1.86 (m, 2H, CH_2), 2.21-2.29 (m, 2H, CH_2), 3.35-3.40 (m, 1H, CH), 6.08 (d, J = 9.2 Hz, 1H, CH), 6.93 (s, 1H, NH), 7.23 (t, J = 8.0 Hz, 1H, H-Ar), 7.32-7.38 (m, 5H, H-Ar). ^{13}C-NMR (125 MHz, $CDCl_3$) δ, ppm: 23.9, 29.6, 30.2, 54.9, 66.2, 123.0, 128.0, 128.4, 129.3, 130.0, 133.4, 133.6, 134.2, 135.0, 135.8, 159.0 and 177.1. CHN Elemental analysis for **$C_{21}H_{17}Cl_4N_3S$**: Calculated: C, 51.98; H, 3.53; N, 8.66; Found: C, 51.77; H, 3.41; N; 8.41.

(*E*)-7-(2-chloro-6-fluorobenzylidene)-3-(2-chloro-6-fluorophenyl)-3,3a,4,5,6,7-hexahydro-2*H*-indazole-2-carbothioamide, 10

Yellow solid; yield: 0.77 g (64.5 %). IR (ν, cm^{-1}): 3260, 3430 (N-H stretch), 3146 (alkene Csp^2-H stretch), 3029 (aromatic Csp^2-H stretch), 2941 (alkyl Csp^3-H stretch), 1587 (C=N stretch), 1470 (aromatic C=C stretch), 1230 (C-N stretch), 1052 (C=S stretch), 755 (C-Cl stretch). ^{1}H-NMR (500 MHz, $CDCl_3$) δ, ppm: 1.47-1.55 (m, 2H, CH_2), 1.75-1.84 (m, 2H, CH_2), 1.93-2.00 (m, 2H, CH), 3.61-3.67 (m, 1H, CH), 6.50 (d, J = 11.0 Hz, 1H, CH), 7.00 (s, 1H, NH_2), 7.38-7.47 (m, 6H, H-Ar). ^{13}C-NMR (125 MHz, $CDCl_3$) δ, ppm: 23.6, 25.4, 28.7, 50.4, 64.6, 126.3, 126.7, 126.8, 127.3, 128.8, 129.4, 129.8, 129.9, 130.7, 131.5, 132.1, 133.8, 134.7, 134.9, 160.7, and 176.2. CHN Elemental analysis for **$C_{21}H_{17}Cl_2F_2N_3S$**: Calculated: C, 55.76; H, 3.79; N, 9.29; Found: C, 54.53; H, 3.27; N; 9.01.

2.2 Materials and methods for the cytotoxic study

2.2.1 Cell Culture

The human breast cancer (MCF-7) and non-cancerous (MCF-10A) cell lines were originally obtained from the American Type Culture Collection (ATCC, USA). MCF-7 cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. MCF-10A cell line was cultured in a mixture of DMEM and Ham's F-12 (DMEM/F12) medium, containing 5% (v/v) horse serum, 10 µg/mL insulin, 20 ng/mL human epidermal growth factor (hEGF), 0.5 µg/mL hydrocortisone and 0.01% (v/v) penicillin-streptomycin antibiotics. All the cell lines were routinely cultured and maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2.2 Cell viability assay

MCF-7 cancer cell lines were seeded at a density of 1×10^4 cells/well in a 96-well plate. MCF-10A was used as a control cell line to determine the selectivity index (SI) values[21]. The cells were seeded and allowed for attachment overnight and subsequently treated using fresh assay medium supplemented with the presence of increasing concentrations of synthesized compounds (0-100 µg/mL) within 24-72 hours of incubation periods at 37 ℃. Standard chemotherapeutic drugs including tamoxifen and cisplatin were used as the positive control, while medium alone was used as the negative control (untreated). At each incubation period, 10 µL (5 mg/mL) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added into each well, and incubated for 4 h at 37 ℃, 5% CO_2. Following this, the MTT solution was discarded and replaced with 100 µL of dimethyl sulfoxide (DMSO) into each well for crystal solubilization. The absorbance of each sample was measured in a microplate reader at 570 nm

wavelength with a reference wavelength of 620 nm according to the manufacturer's protocol.
Half-maximal inhibitory concentration (IC_{50}) values for all cell lines were determined based on the following equation (1).

% viable cells = [(Abs_{sample} – Abs_{blank}) / ($Abs_{untreated}$ / Abs_{blank}) x100] … Equation (1)

Abs: Absorbance reading at 570 nm
Sample: Respective compounds or chemotherapeutic drug
Blank: Culture medium alone

The selectivity index (SI) values were calculated as the ratio of the 50% cytotoxic concentration on the control cell line (IC_{50} in non-cancerous cell line) to the 50% cytotoxic concentration (IC_{50} in cancer cell line), as shown in equation (2).

SI value = IC_{50} in non-cancerous cell line / IC_{50} in cancer cell line …. Equation (2)

2.3 Molecular Docking

2.3.1 Protein preparation

The X-ray crystal structure of the ERα was downloaded from the RCSB database (PDB ID: 3ERT) [1]. Biovia Discovery Studio Visualizer 16.1 was utilized to remove the heteroatoms, water, and prepare the protein further [22].

2.3.2Ligand preparation

Ten synthesized compounds were used as the ligands, as well as Tamoxifen, which was selected as a control reference in the docking studies. The 2D chemical structure of the ligands was built using PerkinElmer ChemDraw software 16.0. Then, the sketched ligands were subjected to energy minimization (MM2 force field) using PerkinElmer Chem3D 16.0 and saved in PDB format.

2.3.3 Docking and Scoring Protocol

AutoDock 4.2 is a computational software used to prepare the ligands and protein, as well as to generate the docking process [23,24]. A click-by-click protocol was used to enforce this process [25,]. Initially, the polar hydrogens and Kollman charges were added to the ERα. Then, the selected ligands were revitalized by Gasteiger charges. The size of the grid box was set to 50*50*50, and the coordinates were 31.6615, -0.8435, 25.1743 (as x, y, z, respectively) with a spacing of 0.375. For the docking parameter, the ERα was defined as rigid while all ligands were flexible. The genetics algorithm run was set to 100, and the Lamarckian genetic was selected to proceed with the docking, while the remaining parameters were kept default. Docking scores were interpreted using Discovery Studio Visualizer 16.1 and LigandScout 4.3 academic license, so that the ionic bonds, hydrogen bonds, and hydrophobic interactions could be easily observed. [22-24].

3.0 Result and Discussion

3.1 Cytotoxicity activity of the synthesized compounds

All the synthesized compounds were evaluated for their cytotoxic activity against MCF-7 cancer cell line as well as MCF-10 non-cancerous cell line. The IC_{50} values of the compounds were obtained at three different incubation periods of 24, 48 and 72 hours. The results are summarized in Table 3. In the present study, the degree of selectivity of the synthetic compounds is expressed as per previous reports [21] with a minor modification, as shown in Equation (1).

$$SI = \frac{IC_{50} \text{ of the synthetic compound in a normal cell line}}{IC_{50} \text{ of the same pure compound in the cancer cell line}} \quad \text{….. Equation (1)}$$

The higher the SI, the more promising a compound holds, due to its selectivity for the cancer cells. The SI values below 2.0 indicated that a compound that possessed strong growth inhibitory activity may be toxic because it also kills the normal cells.

Table1: IC_{50} values and selectivity index of bis-chalcone compounds, **1-6** and fused-pyrazoline compounds, **7-10**

Compounds	*Incubation periods*	*IC_{50} values*		*Selective Index (IC_{50} in normal cells/IC_{50} in cancer cells)*
		MCF-7	**MCF-10A**	**MCF-7**
1	24 h	9.5	87.5	**9.21**
	48 h	8	98.5	**12.32**
	72 h	7	78.2	**11.17**
2	24 h	14	100	**7.14**
	48 h	44	>100	-
	72 h	75.5	53	0.71
	24 h	>100	>100	-

3	48 h	>100	74	-
	72 h	24	26	1.08
	24 h	14	100	**7.14**
4	48 h	9.5	39	**4.11**
	72 h	21	24	1.14
	24 h	>100	>100	-
5	48 h	>100	>100	-
	72 h	>100	49.5	-
	24 h	40	>100	-
6	48 h	31.5	48.5	1.54
	72 h	23	33	1.43
	24 h	21.5	41.5	1.93
7	48 h	10.2	56	**5.49**
	72 h	10	69	6.9
	24 h	63.5	49	0.77
8	48 h	17	39	**2.29**
	72 h	16.5	26	4
9	24 h	58	>100	-
	48 h	100	100	1
	72 h	>100	100	-
	24 h	37	37.5	1.01
10	48 h	8.5	55	**6.47**
	72 h	9	59	**6.56**
tamoxifen	**48**	**9.3±0.44**	**23.71±0.99**	**2.54**

(>100) refers to IC_{50} could be beyond the tested concentration (> 100 µM). (-) refers to Selective Index (SI) value could not be determined since the IC_{50} is not established/ beyond than tested concentration (> 100 µM)

Among the bis-chalcone series **1-3** of cyclopentanone, compound **1** exhibited the most potent cytotoxic effect against the MCF-7 cell line, with its IC_{50} values lower than 10 µM at all three different incubation periods. Besides, it was found to be the most selective among all the synthesized compounds towards MCF-7 breast cancer cells due to the high values of the selectivity index obtained (SI = 9.21-12.32). Similarly, among the bis-chalcone series **4-6** of cyclohexanone unit, compound **4** with one chloro substituent at the *ortho* position of the aromatic rings was the most cytotoxic and selective against the MCF-7 cell line. Its IC_{50} value was as low as 9.5 µM after 48 hours of incubation period and its SI value reached 7.14 at an incubation period of 24 hours. Based on these results, it can thus be deduced that the mono-substitution of a chlorine atom at the *ortho* position of the aromatic rings enhanced the cytotoxic activity and selectivity of the bis-chalcone series against the MCF-7 cell line. However, the insertion of another halogen substituent at the *ortho* position of the aromatic rings reduced both the inhibitory activity and selectivity towards MCF-7 cancer cells.

The comparison of pyrazoline derivatives with chalcone compounds showed that fused-pyrazoline derivatives, **7-10** were more potent than the corresponding bis-chalcone, **2-3** and **4-5** against MCF-7 breast cancer cell line. Both fused-pyrazoline compounds **7** and **10** showed significant cytotoxic activity against MCF-7 breast cancer cell line, with their IC_{50} values around 10 µM after 48 and 72 hours of incubation. They were five to seven times more selective towards MCF-7 cancer cells compared to MCF-10A non-cancerous cells, making them the potential anticancer drugs.

3.2 Molecular Docking

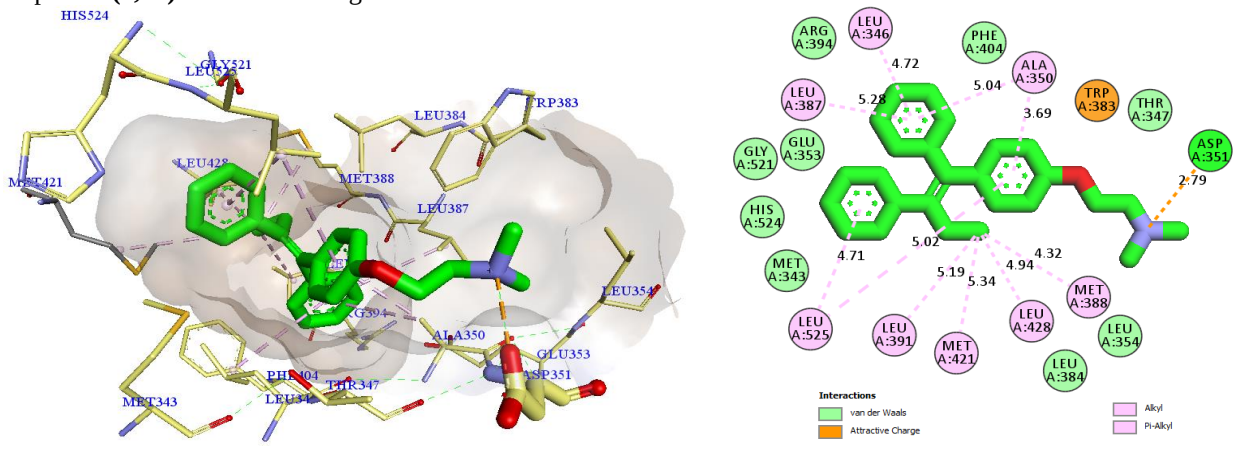
The behavior of all the 10 compounds has been compared with the tamoxifen. To be an effective drug, a compound must have optimum solubility of both water and fat, pass through the intestine, and be transported in aqueous blood before penetrating the cell membrane. Water solubility depends on the number of hydrogen bond donors relative to the alkyl side chain in the compound. Low water solubility means slow absorption and action. Too many hydrogen bond donors contribute to low-fat solubility in which leads to the inability of the drug to cross the cell membrane. A simple method to evaluate the drug-like properties of a compound is to check the compliance with Lipinski's rule (rule of 5) which specifies the numbers of hydrophilic groups, molecular weight, and hydrophobicity. Lipinski's rule of five theorize that an active oral drug should have (i) not more than 5 hydrogen bond donors (OH and NH groups); (ii) not more than 5 hydrogen bond acceptors (notably N and O); (iii) molecular weight less than 500 g/mol; and (iv) octanol-water partition coefficient (log P) less than 5, as summarized in Table 2.

Table 2: Chemical properties based on Lipinski's rule (rule of 5), free binding energy (F.B.E) with the inhibition constant (Ki) of all chalcone derivatives, **1-6**; fused- pyrazoline, **7-10** and the tamoxifen (control)

Compound	*M.W (g/mol)*	*Log P*	*H-bond donor*	*H-bond acceptor*	*F.B.E (Kcal/mol)*	*Inhibition Constant, K_i (nM)*
1	329.22	5.34	0	0	-9.84	60.88
2	398.10	6.45	0	0	-9.42	124.61
3	365.20	5.65	0	0	-9.70	77.37
4	343.25	5.76	0	0	-8.91	292.37
5	412.13	6.87	0	0	-9.30	153.46
6	379.23	6.07	0	0	-8.66	449.45
7	471.22	6.16	1	2	-9.81	64.92
8	438.32	5.36	1	2	-9.27	159.08
9	485.25	6.58	0	1	-8.35	754.76
10	452.34	5.78	0	0	-12.19	1.16
Tamoxifen	371.51	6.07	0	2	-10.40	3.30

Table 2 displays the computed scores of docking between the estrogen receptor alpha (ERα) structure (receptor) and ten synthesized compounds (ligands and the tamoxifen scaffold, the green color represents the carbon atoms, red for oxygen and sky blue for nitrogen atom.). The more negative value shows a high probability of interaction between the ligand and the receptor. As expected, all the ten chalcone derivatives entered the ERα pocket as the tamoxifen possessed varying scores with the enclosed amino acids. All the tested compounds have shown a strong affinity towards ERα with the free binding energy ranging from –12.19 to -8.35 kcal/mol. Remarkably, compound **10** showed the free binding energy of -12.19 kcal/mol, which is much better than the other derivatives. Both compounds**(1,10)** formed a strong interaction with ERα, similar to tamoxifen. The results also showed that the inhibition constant (Ki) of compounds **1** and **10** was roughly the same as that of tamoxifen. The hydrophobic interactions on the tamoxifen have occurred with aromatic rings and the butenyl group. A salt bridge interaction (H-bond and ionic bond) was observed between the tertiary amine and the carboxylic acid group in ASP351, which was considered to be the strongest form of interaction contributing to the stability inside the pocket, as shown in Figure4.

In general, the new design of chalcone and fused-pyrazoline derivatives has been proceeded by the addition of a variety of halogens for aromatic rings, based on the main interactions between tamoxifen and ERα, as shown in Figure 11.



(a) (b)

Figure 4: The docked pose of tamoxifen in the binding site of estrogen receptor alpha (ERα). Using Biovia Discovery Studio Visualizer 16.1 to generate (a) 3D docked interaction and (b) 2D interaction

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Table 3: Summary of the best predicted binding pose of compounds **1** and **10**

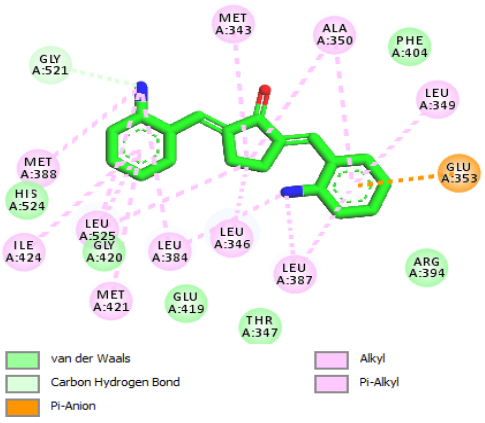
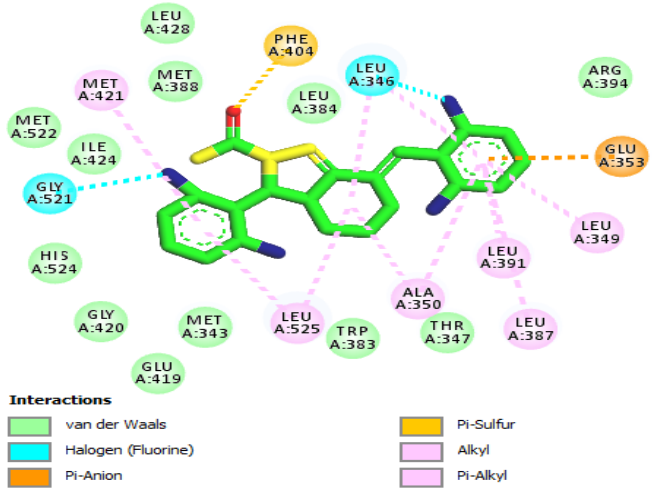
2D-Molecular structure Interaction with 3ERT	Hydrophobic Interaction
 Chalcone **1**	LEU384, ALA350, LEU387, LEU349, GLU353, LEU346, MET343, MET388, LEU525, ILE424, MET524
 Fused-pyrazoline **10**	PHE404, LEU356, GLU353, LEU349, LEU387, LEU391, ALA350, LEU525, GLY524, MET421

Table 3 provides a summary of the best predicted binding pose of chalcone, **1** and fused-pyrazoline, **10** derivatives. The predictions have shown that the aromatic ring in compounds **1** and **10** occupy the ligand-binding domain of ERα in the same way as tamoxifen. In compounds **1** and **10** scaffolds, the green color represents the carbon atoms, red for oxygen, sky blue for chloro and fluro, and yellow for the nitrogen atom.

Conclusion

A series of six bis-chalcones**(1-6)** were successfully synthesized between halogen-substituted benzaldehyde of cyclopentanone and cyclohexanone. The dihalogen-substituted bis-chalcones, **2-3** and **5-6** were chosen for further cyclo-condensation reactions with thiosemicarbazide to form four new fused-pyrazoline derivatives, **7-10**. All the compounds were characterized using FT-IR, ^{1}H and ^{13}C NMR spectroscopy. The cytotoxic activity of all the synthesized compounds was evaluated against MCF-7 breast cancer cell lines as well as MCF-10 non-cancerous epithelial cell lines. The selectivity index values of all the compounds were calculated based on their IC_{50} values towards non-cancerous and cancer cells. Compounds **1**, **4**, **7, 8** and **10** were found to have the IC_{50} values about 20 µM and selectivity index values ranging from 4.11-12.32 against MCF-7 cell line. These compounds are good candidates to be selected for further studies to develop anticancer drugs. The structure-activity relationships study showed that bis-chalcone compounds with mono-substituted chlorine atom at the *ortho* position of their aromatic rings exhibited greater cytotoxic activity and selectivity against MCF-7 cell line compared to those with disubstituted halogen atoms. Moreover, in the cytotoxic activity test towards MCF-7 breast cancer cells, fused-pyrazoline derivatives were found to be more potent than their corresponding bis-chalcone compounds. Analysis of the molecular docking showed that compound with a larger structure has an

increasing number of bonds, leading to more interactions with the residues of amino acids in the active ERα site, which could have enhanced the antitumor activity. Furthermore, the docking scores showed the binding energies of the synthesised compounds 1and 10 to the ERα receptor were the best of all the synthesised derivatives. These findings were broadly compatible with the observed *in vitro* assay investigation. Therefore, further investigation on the molecular modelling and docking studies using AutoDock1.5.6 tools can proceed to predict the best structure with the highest affinity to bind to the breast cancer cell line. Various interactions between the designed compounds (ligands) and receptor active sites will be used to synthesised more new chalcone and fused pyrazoline derivatives for anticancer drugs in the future.

Author contributions

Conceptualization and planning, M.K.; Docking studies, synthesis and characterization, M.M.A., T.Z.N., N.H.S., M.K.; Cytotoxity work, N.N.S.N.M.K. and M.M.; Supervision, M.K.; writing-original draft, M.M.A., M.K.; writing-review and editing, M.K., M.M.A.

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