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Synthesis and Anti-breast Cancer Activity Evaluation of the Designed 3-Chlorobenzo[*b*]thiophene Derivatives: Promising Estrogen Receptor Alpha Inhibitors

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ABSTRACT

Breast cancer is the second leading cause of death in women. Fulvestrant and tamoxifen (TAM) are the most extensively used medications to treat breast cancer. However, these medications are implicated in pharmacokinetics and resistance issues. This research aims to overcome these issues in selectivity and side effects. The study was conducted to designing potent anti-breast cancer agents using benzo[*b*]thiophene (BT) as a building block and then testing the synthesized compounds against breast cancer cell lines (MCF-7). Techniques such as melting point (m.p.), Infrared Spectroscopy (IR), and Nuclear Magnetic Resonance (NMR) used to characterize these compounds (3a-d & 4a-d) and their intermidates. Benzothiophene (BT) scaffold was incorporated to whether thiazolidin-4-one (THZ) or azetidin-2-one (AZT) ring system using the cyclocondensation reaction between the schiff base and ethyl thioglycolate or acetyl chloride to afford the target compounds (3a-d & 4a-d) in a good yield. All the newly synthesized compounds were screened for their in vitro anti-breast cancer activity using the MCF-7 cell line. Compounds (3a) and (3d) revealed promising anti-breast cancer activity with IC_{50} equal 10.32µM and 11.35µM, respectively. Whereas the reference TAM showed IC_{50} at about 18.02µM. This work generated fresh insight that compounds (3a) and (3d) could be considered as a lead candidate to fight breast cancer.

Keywords: breast cancer; estrogen receptor; benzothiophene; tamoxifen; raloxifene; Azetidinone and thiazolidinone.

1. INTRODUCTION

Heterocycles rings are a pivotal role in drug discovery as either scaffolds or pharmacophoric elements to fight cancer. Breast cancer is the most prevalent type of cancer in women and the second leading cause of death.[1] Three major groups of medicines are used as the standard of therapy for individuals with ER+ breast cancer. Those that specifically target estrogen receptor (ER), aromatase inhibitors (AIs) that lower estrogen levels, and cell-cycle checkpoint (CDK4/6) inhibitors (i.e. cyclin-dependent kinases 4 and 6) such as palbociclib.[2] Fulvestrant was first licensed as a complete ER antagonist in the early 2000s, but it was later discovered to be a selective estrogen receptor degrader (SERD). However, because of its poor druglike qualities, Fulvestrant must be administered intramuscularly, i.e. low solubility, which limits its target occupancy and, as a result, its efficacy.[2, 3] Tamoxifen, is a selective estrogen receptor modulator (SERM), was licensed in the 1970s. It is evident that tamoxifen works as a partial agonist, increasing its affinity to cause endometrial cancer and its implication in the development of breast cancer resistance. [2] Around 90% of tumours preserve or express ER in the resistance scenario, where ER offers prosurvival signalling even in the absence of estrogens.[3, 4]

Benzo[*b*]thiophene (BT) is an interesting heterocycle that offers diverse biological and pharmacological properties to treat breast malignancy. Pharmaceutical medications like raloxifene (Ral) and arzoxifene (Arz) have benzothiophene (BT) in their chemical structures

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(Fig.1). Raloxifene has been used for decades in treating breast cancer and postmenopausal osteoporosis. It belongs to the selective estrogen receptor modulators (SERMs) class of drugs, which have estrogen agonist-like effects on bone tissues and serum lipids while having significant estrogen antagonist characteristics in the breast and uterus.[5] Its benzothiophene (BT) scaffold has been approved for use in the development of new ER-targeted ligands.[5, 6] Various of chemical changes have been carried out to enhance potency and pharmacokinetic (PK) qualities (low bioavailability), resulting in a variety of ER ligands with acceptable action. Arzoxifene, G1T48 (G1), and LSZ-102 (Novartis) have all been shown to be effective antineoplastic drugs in breast cancer models (Fig. 1).[7-10] This research is conducted by designing and synthesis the novel compounds using benzothiophene (BT) as a core building block and finally testing these compounds by in vitro cell line (MCF-7) against estrogen (+) breast cancer.

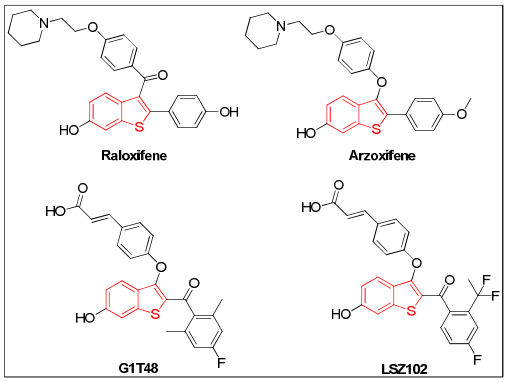


Fig. 1: Benzothiophene-based structures as estrogen receptor ligands

2. EXPERIMENTAL SECTION

2.1 *Chemicals and Reagents*

All analytical grade reagents and anhydrous solvents used exactly as they were purchased from commercial vendors (U.K., Spain, Germany, China, and India). The Hyper-Chem Company in the China and Florochem in the UK provided 3-chlorobenzo[*b*]thiophene-2- carbonyl chloride. Benzaldehyde derivatives, acetyl chloride and ethyl thioglycolate, supplied from Hangzhou Hyper Chemicals Limited. Ethylene diamine purchased from Central Drug House (P) Ltd – CDH. The commercially available solvents and chemicals used without purification. A graduated cylinder was used to measure the large volume of solvent and the syringe used for a small once. Acetone was used to wash the glassware and pre-heated oven for complete drying.

2.2 *Instrumentation*

Stuart equipment *SMP3* (U.K.) was used to calculate melting points through the open capillary method. IR spectra were recorded on KBr disks/ ATR using a Shimadzu FT-IR spectrophotometer. ^{1}H-NMR spectra (Burker DMX -500 NMR spectrophotometer) were recorded on ultra-shield 500 MHZ in Iran, with tetramethylsilane (TMS) as the internal standard and DMSO_d_6 as a solvent. ^{13}C NMR spectra were collected on a Bruker (100 MHz) and Varian (126 MHz) spectrometer in Iraq and Iran, respectively.

Using trypsin/EDTA, RPMI 1640, and fetal bovine serum (Fisher Scientific, USA), a cytotoxicity experiment was performed at the tissue culture centre/ pharmacology and toxicology department/ College of Pharmacy, Mustansiriyah University. MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl – 2Htetrazoliumbromide] (Fisher Scientific, USA); dimethyl sulfoxide (DMSO) (Santacruz Biotechnology, Dallas, TX, USA); phosphate-buffered saline (PBS) (Gibco, USA); CO_2 incubator with laminar flow hood (Memert, Germany); autoclave (Astell, Germany); freezer -20℃ (Crafft, Korea); freezer -80℃ (Crafft, Korea); (Gel, Germany).

2.3 *General Procedure for the synthesis of N1-benzylideneethane-1,2-diamine derivatives (1a-d)*

To a 100 mL round bottom flask, ethane-1,2-diamine (0.01 mole) was dissolved in EtOH (20 mL) while stirring. Then, benzaldehyde derivatives (0.01 mole) were dissolved in EtOH (10 mL) and added dropwise over 30 minutes. Then, the reaction was left on reflux at 83℃ for 7 hrs. After that, the clear mixture was cooled in the ice bath, and white powder showed up. The mixture was filtered and recrystallized using EtOH (25 mL) and filtered. [11-13]

*N*1-(4-methoxybenzylidene)ethane-1,2-diamine (1a)

Chemical Formula:$C_{10}H_{14}N_2O$. Molecular Weight: 178.2 g/mol. Physical properties: White crystals (91% yield); m.p.(144-146)℃. FT-IR (υ) cm^{-1}: 3267, 3200 (NH_2), 2970- 2839 (C-H; aliphatic), 1597 (C=N), 1504 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.62 (s, 1H, benzylic C-H), 7.66 (d, 2H, ortho to -OMe), 6.98 (d, meta to -OMe), 3.86 (m, 6H, overlapped two aliphatic –CH_2 and –NH_2), 3.36 (s, 3H, -OMe). ^{13}C NMR (126 MHz, DMSO_d_6) δ (ppm): 161.60 (MeO-C, Ph), 161.51 (C=N, Schiff base), 129.85 (2C, meta to OMe, Ph), 129.41 (C-C=N, Ph), 114.48 (2C, ortho to OMe, Ph), 61.50 (C=N-C), 55.72 (O-CH_3), 40.50 (C-NH_2).

*N*1-(4-chlorobenzylidene)ethane-1,2-diamine (1b)

Chemical Formula:$C_9H_{11}ClN_2$. Molecular Weight: 182.7 g/mol. Physical properties: Pearl crystals (90% yield); m.p. (148-150)℃. FT-IR (υ) cm^{-1}: 3275, 3225 (NH_2), 2941- 2854 (C-H; aliphatic), 1602 (C=N), 1513 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.33 (s, 1H, benzylic C-H), 7.72-7.70 (m, 2H, ortho to Cl), 7.47 (d, 2H, meta to Cl), 3.86 (s, 4H, two aliphatic –CH_2), 2.49 (s, 2H, NH_2). ^{13}C NMR (126 MHz, DMSO_d_6) δ (ppm): 161.17 (C=N, Schiff base), 142.09 (Cl-C, Ph), 140.72 (C-C=N, Ph), 129.77 (2C, meta to Cl, Ph), 129.10 (2C, ortho to Cl, Ph), 57.54 (C=N-C), 41.47 (C-NH_2).

***N*1-(4-methylbenzylidene)ethane-1,2-diamine (1c)**

Chemical Formula: $C_{10}H_{14}N_2$. Molecular Weight: 162.2 g/mol. Physical properties: White ppt. (88% yield); m.p. (174-176) ℃. FT-IR (υ) cm^{-1}: 3275, 3240 (NH_2), 2910- 2850 (C-H; aliphatic), 1597 (C=N), 1512 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6): δ_H (ppm) 8.62 (s, 1H, benzylic C-H), 7.57 (d, 2H, ortho to CH_3), 7.21 (d, 2H, meta to CH_3), 3.82 (s, 2H, aliphatic –CH_2), 3.35 – 3.29 (m, 2H, aliphatic CH_2), 3.31 (s, 2H, NH_2), 2.30 (s, 3H, Ar-CH_3). ^{13}C NMR (100 MHz, DMSO_d_6) δ (ppm) : 162.17 (C=N, Schiff base), 140.82 (CH_3-C, Ph), 133.99 (C-C=N, Ph), 129.66 (2C, meta to Me, Ph), 128.24 (2C, ortho to Me, Ph), 61.38 (C=N-C), 43.73 (C-NH_2), 21.46 (CH_3-Ph).

***N*1-(4-nitrobenzylidene)ethane-1,2-diamine (1d)**

Chemical Formula: $C_9H_{11}N_3O_2$. Molecular Weight: 193.2 g/mol. Physical properties: Brown ppt. (91% yield); m.p. (169-170)℃. FT-IR (υ) cm^{-1}: 3441, 3356 (NH_2), 2981- 2927 (C-H; aliphatic), 1620 (C=N), 1454 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.51 (s, 1H, benzylic C-H), 8.28-8.26 (m, 2H, ortho to NO_2), 7.98-7.95 (m, 2H, meta to NO_2), 3.98 (s, broad, 4H, two aliphatic -CH_2). 3.33 (s, 2H, -NH_2). ^{13}C NMR (126 MHz, DMSO_d_6) δ (ppm): 161.17 (C=N, Schiff base), 149.05 (CH_3-C, Ph), 143.75 (C-C=N, Ph), 129.30 (2C, meta to NO_2, Ph), 124.39 (2C, ortho to NO_2, Ph), 61.15(C=N-C),40.49 (C-NH_2).

2.4 General Procedure for the synthesis of N1-(2-(benzylideneamino)ethyl)-3-chlorobenzo[b]thiophene-2-carboxamide derivatives (2a-d)

To a 100 mL round bottom flask, one of the compounds (1a-d) (0.001 mol) was dissolved in 1,4 dioxane (15 mL) with stirring. Then, 3-chlorobenzo[*b*]thiophene-2-carbonyl chloride (0.001 mol.) was dissolved in 1, 4-dioxane (10 mL) was added dropwise over 30 minutes. The ppt. was showed up, and the mixture was left for reflux for 6 hrs at 86℃. After that, the mixture was cooled in an ice bath and off-white ppt. was showing up. The mixture was filtered and recrystallized using EtOH (60 mL) and filtered.[14, 15]

3-Chloro-*N*-(2-((4-methoxybenzylidene)amino)-ethyl) benzo[*b*]thiophene-2-carboxamide (2a)

Chemical Formula: $C_{19}H_{17}ClN_2O_2S$. Molecular Weight: 372.9 g/ mol. Physical properties: Off white creamy ppt. (64% yield); m.p.(249-251)℃. FT-IR (υ) cm^{-1}: 3282 (N-H), 3051 (C-H; aromatic), 2939, 2893 (C-H; aliphatic), 1654 (C=O), 1624 (C=N), 1510 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.52 (s, 2H, overlapped N-H and benzylic C-H), 8.10 (m, 2H, ortho to OMe), 7.91 (m, 2H, meta to OMe), 7.61 – 7.54 (m, 4H, benzothiophene), 3.58-3.53 (m, broad, 4H, two aliphatic –CH_2), 3.31 (s, 3H, OMe). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 160.93 (O=C-NH), 160.64 (C=N, Schiff base), 141.60 (S-C-C=O, BT), 138.76 (MeO-C, Ph), 137.14(C-C=N, Ph), 127.95 (C-Cl, BT), 126.42 (2C, meta to OMe, Ph), 123.87 (2C, ortho to OMe, Ph), [136.51, 132.85, 123.04, 121.17, 119.28 (6C, benzene ring of BT)], 58.49 (O-CH_3), 54.79 (C=N-C, aliphatic), 40.45 (C-NH-CO, aliphatic).

3-Chloro-*N*-(2-((4-chlorobenzylidene)amino)-ethyl) benzo[*b*]thiophene-2-carboxamide (2b)

Chemical Formula: $C_{18}H_{14}C_{l2}N_2OS$. Molecular Weight: 377.3g/ mol. Physical properties: Off white creamy ppt. (67% yield); m.p. (254-255)℃. FT-IR (υ) cm^{-1}: 3286 (N-H), 3051 (C-H; aromatic), 2939, 2850 (C-H; aliphatic), 1620 (C=O) & (C=N), 1550 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.52 (s, 2H, overlapped N-H and benzylic C-H), 8.10 (dd, 2H, ortho to NO_2), 7.91 – 7.85 (m, 2H, meta to NO_2), 7.61 – 7.54 (m, 4H, benzothiophene), 3.58-3.53 (m, broad, 4H, two aliphatic –CH_2).^{13}C NMR (100 MHz, DMSO_d_6) δ (ppm): 160.94 (O=C-NH), 160.34 (C=N, Schiff base), 148.06 (S-C-C=O, BT), 142.62 (Cl-C, Ph), 137.16 (C-C=N, Ph), 127.94 (C-Cl, BT), 126.40 (2C, meta to Cl, Ph), 123.86 (2C, ortho to Cl, Ph), [136.52, 132.86,123.03, 121.56, 119.28 (6C, benzene ring of BT)], 59.95 (C=N-C, aliphatic), 48.94 (C-NH-CO, aliphatic).

3-Chloro-*N*-(2-((4-methylbenzylidene)amino)-ethyl)benzo[*b*]thiophene-2-carboxamide (2c)

Chemical Formula: $C_{19}H_{17}ClN_2OS$. Molecular Weight: 356.9g/ mol. Physical properties: White flaky ppt. (61% yield); m.p. (252-254) ℃. FT-IR (υ) cm^{-1}: 3286 (N-H), 3055 (C-H; aromatic), 2939 & 2845 (C-H; aliphatic), 1624 (C=O) & (C=N), 1546 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.51 (s, 2H, overlapped N-H and benzylic C-H), 8.11 (m, 2H, ortho to Me), 7.84 (m, 2H, meta to Me), 7.56 (m, 4H, benzothiophene), 3.99 (m, 4H, two aliphatic –CH_2), 2.29 (s, 3H, Me). ^{13}C NMR (126 MHz, DMSO_d_6) δ (ppm): 160.33(O=C-NH), 159.32(C=N, Schiff base), 144.64 (S-C-C=O, BT),

141.57 (Me-C, Ph), 135.29 (C-C=N, Ph), 126.47 (C-Cl, BT), 123.94(2C, meta to Me, Ph), 122.57 (b, 2C, ortho to Me, Ph), [130.31, 129.53,122.22, 120.27, 119.14, (6C, benzene ring of BT)], 49.47 (C=N-C, aliphatic), 43.78 (C-NH-CO, aliphatic), 24.23 (Ar-CH_3).

3-Chloro-*N*-(2-((4-nitrobenzylidene)amino)-ethyl)benzo[*b*]thiophene-2-carboxamide (2d)

Chemical Formula: $C_{18}H_{14}ClN_3O_3S$. Molecular Weight: 387.8 g/ mol. Physical properties: Creamy ppt. (58% yield); m.p.(205-206) ℃. FT-IR (υ) cm^{-1}: 3282 (N-H), 3051 (C-H; aromatic), 2939 & 2893 (C-H; aliphatic), 1654(C=O), 1624 (C=N), 1510 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.54 (s, 2H, overlapped N-H and benzylic C-H), 8.10 (dd, 2H, ortho to NO_2), 7.91 – 7.86 (m, 2H, meta to NO_2), 7.61 – 7.54 (m, 4H, benzothiophene), 3.56 (s, broad, 4H, two aliphatic –CH_2). ^{13}C NMR (126 MHz, DMSO_d_6) δ (ppm): 162.52 (h, O=C-NH), 162.50 (C=N, Schiff base), 144.47 (S-C-C=O, BT), 140.08 (NO_2-C, Ph), 138.10 (C-C=N, Ph), 128.64 (C-Cl, BT), 125.19 (2C, meta to NO_2, Ph), 123.95(2C, ortho to NO_2, Ph), 136.80, [135.33,122.51, 119.11, 109.99, (6C, benzene ring of BT)], 58.89 (C=N-C, aliphatic), 49.24 (C-NH-CO, aliphatic).

2.5 General Procedure for the synthesis of 3-chloro-N-(2-(4-oxo-2-phenylthiazolidin-3-yl)ethyl) benzo[b] -thiophene-2-carboxamide derivatives: (3a-d)

To a 100 mL round bottom flask, one of the compounds (2a-d) (0.01 mol) was dissolved in dry 1,4 dioxane (20 mL) and left stirring in a water bath at 70℃. Then, a pinch of anhydrous zinc chloride was added and left on stirring for 2 hrs. After that, ethyl thioglycolate (0.012 mol) was dissolved in dry 1, 4 dioxane (5 mL). The reaction mixture was left stirring on reflux at 70℃ for 12 hr. Then, the reaction mixture was quenched by pouring it into the crushed pieces of ice, filtered, left to dry and recrystallized from rectified ethanol to give a powder.[16-20]

3-Chloro-*N*-(2-(2-(4-methoxyphenyl)-4-oxothiazolidin-3-yl)ethyl)benzo[*b*]thiophene-2-carboxamide (3a)

Chemical Formula: $C_{21}H_{19}ClN_2O_3S_2$. Molecular Weight: 447.0 g/ mol. Physical properties: white powder. (38% yield); m.p.(254-256) ℃. FT-IR (υ) cm^{-1}: 3286 (N-H), 3055 (C-H; aromatic), 2939-2840 (C-H; aliphatic), 1624 (C=O), 1550 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.53 (s, 2H, N-H and benzylic C-H), 8.13-8.07 (m, 2H, Ar-H ortho to OMe), 7.91- 7.86 (m, 2H, Ar-H meta to OMe), 7.59- 7.56 (m, 4H, benzothiophene-H), 3.57-3.53 (m, 6 H, two aliphatic -CH_2 and thiazolidinone –CH_2), 3.31 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, DMSO_d_6) δ (ppm): 170.20 (O=C-N, THZ), 160.95(O=C-NH), 141.38 (S-C-C=O, BT), 137.14 (MeO-C, Ph), 136.51 (C-C=N, Ph), 127.96 (C-Cl, BT), 126.42 (2C, meta to OMe, Ph), 123.85 (2C, ortho to Cl, Ph), [132.83, 123.03, 121.86,119.29 (6C, benzene ring of BT)], 72.93 (N-CH-S, benzylic), 54.66 (O-CH_3), 42.50 (C=N-C, aliphatic), 41.85(C-NH-CO, aliphatic), 33.01(CH_2, THZ).

3-Chloro-*N*-(2-(2-(4-chlorophenyl)-4-oxothiazolidin-3-yl)ethyl)benzo[*b*]thiophene-2-carboxamide (3b)

Chemical Formula: $C_{20}H_{16}Cl_2N_2O_2S_2$. Molecular Weight: 451.4 g/ mol. Physical properties: white powder. (42% yield); m.p.(254-255) ℃. FT-IR (υ) cm^{-1}: 3286 (N-H), 3055 (C-H; aromatic), 2939-2845 (C-H; aliphatic), 1624 (C=O), 1546 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.52 (s, 2H, N-H and benzylic C-H), 8.10 (m, 2H, Ar-H ortho to Cl), 7.88 (m, 2H, Ar-H meta to Cl), 7.57 (m, 4H, benzothiophene-H), 3.55 (dt, 6H, two aliphatic -CH_2 and thiazolidinone –CH_2). ^{13}C NMR (126 MHz, DMSO_d_6) δ (ppm): 169.20 (O=C-N, THZ), 160.90 (O=C-NH), 154.78 (2C, S-C-C=O, BT & C-C=N, Ph), 144.28 (Cl-C, Ph), 132.84 (C-Cl, BT), 127.91 (2C, meta to Cl, Ph), 126.38 (2C, ortho to Cl, Ph), [137.13, 134.78, 123.84, 123.01, (6C, benzene ring of BT)], 69.57 (N-CH-S, benzylic), 46.14 (C=N-C, aliphatic), 44.04 (C-NH-CO, aliphatic), 34.75 (CH_2, THZ).

3-Chloro-N-(2-(4-oxo-2-(p-tolyl)thiazolidin-3-yl)ethyl) benzo[b]thiophene-2-carboxamide (3c)

Chemical Formula: $C_{21}H_{19}ClN_2O_2S_2$. Molecular Weight: 431.0 g/ mol. Physical properties: white powder. (33% yield); m.p.(250-251) ℃. FT-IR (υ) cm^{-1}: 3282 (N-H), 3055 (C-H; aromatic), 2935-2850 (C-H; aliphatic), 1620 (C=O), 1546 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.53 (s, 2H, N-H and benzylic C-H), 8.11-8.09 (m, 2H, Ar-H ortho to CH_3), 7.89 (d, 2H, Ar-H meta to CH_3), 7.59-7.57 (m, 4H, benzothiophene-H), 3.56 (m, 6H, aliphatic and thiazolidinone –CH_2), 2.50 (s, 3H, $ArCH_3$). ^{13}C NMR (100 MHz, DMSO_d_6) δ (ppm): 173.31 (O=C-N, THZ), 160.94 (O=C-NH), 142.20 (S-C-C=O, BT),137.14 (Me-C, Ph), 136.51 (C-C=N, Ph), 127.96 (C-Cl, BT), 126.42 (2C, meta to Me, Ph), 123.86 (2C, ortho to Me, Ph), [132.84, 124.87, 123.04, 120.48, 119.29, (6C, benzene ring of BT)], 66.99 (N-CH-S, benzylic), 42.81 (C=N-C, aliphatic), 41.97 (C-NH-CO, aliphatic), 36.59 (CH_2, THZ), 22.66 (CH_3-Ar).

3-Chloro-*N*-(2-(2-(4-nitrophenyl)-4-oxothiazolidin-3-yl)ethyl)benzo[*b*]thiophene-2-carboxamide (3d)

Chemical Formula: $C_{20}H_{16}ClN_3O_4S_2$. Molecular Weight: 461.9 g/ mol. Physical properties: off white powder (67% yield); m.p.(252-253) ℃. FT-IR (υ)

cm^{-1}: 3282 (N-H), 3051 (C-H; aromatic), 2935-2854 (C-H; aliphatic), 1624 (C=O), 1546 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.53 (s, 2H, N-H and benzylic C-H), 8.10 (dd, 2H, Ar-H ortho to NO_2), 7.92- 7.85 (m, 2H, Ar-H meta to NO_2), 7.61 – 7.54 (m, 4H, benzothiophene-H), 3.58-3.54 (m, 4H, two aliphatic $-CH_2$), 3.32 (s, 2H, thiazolidinone $-CH_2$). ^{13}C NMR (100 MHz, DMSO_d_6) δ (ppm): 172.36 (O=C-N, THZ), 160.94 (O=C-NH), 144.32 (S-C-C=O, BT), 137.15 (NO_2-C, Ph), 136.51 (C-C=N, Ph), 126.42 (C-Cl, BT), 126.41 (2C, meta to NO_2, Ph), 123.86 (2C, ortho to NO_2, Ph), [132.85, 127.95,123.04, 123.03, 119.28 (6C, benzene ring of BT)], 68.31(N-CH-S, benzylic), 42.59 (C=N-C, aliphatic), 41.85 (C-NH-CO, aliphatic), 37.33 (CH_2, THZ).

2.6 General Procedure for the synthesis of 3-chloro-N-(2-(2-oxo-4-phenylazetidin-1-yl)ethyl)benzo [b] thiophene-2-carboxamide derivatives: (4a-d)

To a 100 mL round bottom flask, one of the compounds (2a-d) (0.0 1 mol) was dissolved in dry 1,4-dioxane (20 mL), and the mixture was left to heat up until 70℃ on stirring for complete solubility. Then TEA (0.025 mol, 2.5 eq.) was dissolved in a sufficient volume of dry 1,4-dioxane and gently added to the reaction mixture. The mixture was left on stirring while heating in a water bath at 70℃ for (3 hrs). Then, the reaction was cooled to 48℃, and acetyl chloride (0.015 mol) was dissolved in dry 1,4-dioxane (5 mL) and added to the reaction mixture gradually. After that, the reflux was set up, and the reaction mixture was left stirring at 85 ℃ for 6 hrs. Then, the white ppt. of ammonium chloride was filtered off, and the reaction was left on reflux for another 72 hrs. Then, the reaction mixture was quenched by pouring it into the crushed pieces of ice, filtered, left to dry and recrystallized from benzene (5 mL) to give a powder.[18, 21]

3-Chloro-*N*-(2-(2-(4-methoxyphenyl)-4-oxoazetidin-1-yl)ethyl)benzo[*b*]thiophene-2-carboxamide (4a)

Chemical Formula: $C_{21}H_{19}ClN_2O_3S$. Molecular Weight: 414.9 g/ mol. Physical properties: yellow powder (36% yield); m.p. (253-254)℃. FT-IR (υ) cm^{-1}: 3286 (N-H), 3055 (C-H; aromatic), 2935-2840 (C-H; aliphatic), 1620 (C=O), 1531 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.52 (s, 2H, N-H and benzylic C-H), 8.11-8.08 (m, 2H, Ar-H ortho to -OMe), 7.88 (m, 2H, Ar-H meta to -OMe), 7.58 – 7.56 (m, 4H, benzothiophene-H), 3.57 – 3.54 (m, 6H, aliphatic and β-lactam $-CH_2$), 3.31 (s, 3H, OMe). ^{13}C NMR (126 MHz, DMSO_d_6) δ (ppm): 169.59(O=C-N, AZT), 160.91(h, O=C-NH), 148.69 (MeO-C, Ph), 141.58 (S-C-C=O, BT), 137.13 (C-C=N, Ph), 128.76 (C-Cl, BT), 127.91(2C, meta to OMe, Ph), 126.37(2C, ortho to OMe, Ph), [136.50, 132.85,123.84, 123.00, 119.25, (6C, benzene ring of BT)], 69.50 (N-CH-S, benzylic), 65.97(O-CH_3), 51.36 (C=N-CH_2, aliphatic), 47.23 (CH_2, AZT), 37.56 (CH_2-NH-CO, aliphatic).

3-Chloro-*N*-(2-(2-(4-chlorophenyl)-4-oxoazetidin-1-yl)ethyl)benzo[*b*]thiophene-2-carboxamide (4b)

Chemical Formula: $C_{20}H_{16}Cl_2N_2O_2S$. Molecular Weight: 419.3 g/ mol. Physical properties: yellow powder (42% yield); m.p. (259-261) ℃. FT-IR (υ) cm^{-1}: 3290 (N-H), 3055 (C-H; aromatic), 2939-2835 (C-H; aliphatic),1624 (C=O), 1535 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.52 (s, 2H, N-H and benzylic C-H), 8.11-8.08 (m, 2H, Ar-H ortho to Cl), 7.89 (m, 2H, Ar-H meta to Cl), 7.59-7.56 (m, 4H, benzothiophene-H), 3.57 – 3.54 (m, 6H, aliphatic and β-lactam $-CH_2$). ^{13}C NMR (100 MHz, DMSO_d_6) δ (ppm): 167.31(O=C-N, AZT), 160.93(O=C-NH), 140.85(S-C-C=O, BT), 137.14 (Cl-C, Ph), 136.51 (C-C=N, Ph), 127.95 (C-Cl, BT), 126.41 (2C, meta to Cl, Ph), 123.87 (2C, ortho to Cl, Ph), [132.85, 128.79, 123.03, 121.10, 119.28, (6C, benzene ring of BT)], 52.89 (N-CH-S, benzylic), 40.60 (C=N-CH_2, aliphatic), 40.45 (CH_2, AZT), 37.37 (CH_2-NH-CO, aliphatic).

3-Chloro-*N*-(2-(2-oxo-4-(p-tolyl)azetidin-1-yl)ethyl)benzo[*b*]thiophene-2-carboxamide (4c)

Chemical Formula: $C_{21}H_{19}ClN_2O_2S$. Molecular Weight: 398.9 g/ mol. Physical properties: yellow powder (33% yield); m.p.(258-260) ℃. FT-IR (υ) cm^{-1}: 3295 (N-H), 3055 (C-H; aromatic), 2973-2856 (C-H; aliphatic), 1628 (C=O), 1539 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.51 (s, 2H, N-H and benzylic C-H), 8.09 (m, 2H, Ar-H ortho to CH_3), 7.88 (m, 2H, Ar-H meta to CH_3), 7.57 (m, 4H, benzothiophene-H), 3.56 (m, 6H, two aliphatic $-CH_2$ and β-lactam $-CH_2$), 2.50 (s, 3H, Ar-CH_3). ^{13}C NMR (126 MHz, DMSO_d_6) δ (ppm): 171.73 (O=C-N, AZT), 159.63(O=C-NH), 152.57 (Me-C, Ph), 146.67 (S-C-C=O, BT), 139.08 (C-C=N, Ph), 129.28(C-Cl, BT), 127.92 (2C, meta to Me, Ph), 123.38 (2C, ortho to Me, Ph), [136.19, 130.96,121.55, 120.69, 118.53: (6C, benzene ring of BT)], 62.54 (N-CH-S, benzylic), 59.27 (C=N-CH2, aliphatic), 50.82 (CH_2, AZT), 37.93 (CH_2-NH-CO, aliphatic), 22.62 (CH_3-Ar).

3-Chloro-*N*-(2-(2-(4-nitrophenyl)-4-oxoazetidin-1-yl)ethyl)benzo[*b*]thiophene-2-carboxamide (4d)

Chemical Formula: $C_{20}H_{16}ClN_3O_4S$. Molecular Weight: 429.9 g/ mol. Physical properties: Bright yellow crystals (30% yield); m.p.(255-257) ℃. FT-IR (υ) cm^{-1}: 3286 (N-H), 3059 (C-H; aromatic), 2935-2858 (C-H; aliphatic), 1620 (C=O), 1535 (C=C; aromatic). ^{1}H NMR (500 MHz, DMSO_d_6) δ_H (ppm): 8.52 (s, 2H, N-H and benzylic C-H), 8.10 (dd, 2H, Ar-H ortho to NO_2), 7.89 (dd, 2H, Ar-H meta to

NO_2), 7.59 – 7.56 (m, 4H, benzothiophene-H), 3.57-3.54 (m, 6H, two aliphatic -CH_2 and β-lactam –CH_2), 2.07 (s, 2H, OH of NO_2). ^{13}C NMR (100 MHz, DMSO_d_6) δ (ppm): 167.80 (O=C-N, AZT), 160.95 (O=C-NH), 146.00 (NO_2-C, Ph), 139.35 (S-C-C=O, BT), 137.15 (C-C=N, Ph), 127.95 (C-Cl, BT), 126.41 (2C, meta to NO_2, Ph), 123.85 (2C, ortho to NO_2, Ph), [136.51, 132.85, 123.03, 121.47, 119.28: (6C, benzene ring of BT)], 59.45 (N-CH-S, benzylic), 53.16(C=N-CH_2, aliphatic), 40.62 (CH_2, AZT), 37.20 (CH_2-NH-CO, aliphatic).

2.7 In vitro Cytotoxicity Test by MTT assay

To evaluate the capacity of compounds to inhibit MCF-7 cells, an MTT assay was used. The MTT assay measures cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. This colourimetric assay is based on reducing a yellow tetrazolium salt (3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells. The viable cells contain NADPH-dependent oxidoreductase enzymes, which reduce the MTT to formazan.(22)

The insoluble formazan crystals are dissolved using a solubilization solution, and the resulting coloured solution is quantified by measuring absorbance at 500-600 nanometers using a multi-well spectrophotometer.

After cultured MCF-7 cell lines, the cells viewed using an inverted microscope to assess the degree of 80 % confluences and confirm the absence of bacterial and fungal contaminants, then removed and discarded the culture medium. The cell layer rinsed with 5 mL of Dulbecco's PBS without $Ca^{2+/}Mg^{2+}$solution to remove all traces of serum that contains trypsin inhibitor and repeated this step 3 times. The trypsin-EDTA solution (3 mL) has added to the flask to cover the cells and extract gently with a pipette the excess of the trypsin-EDTA solution and leave enough solution to cover the cell monolayer and incubate 3 minutes at 37°C and check periodically for cell detachment, then observed cells under an inverted microscope until cell layer is dispersed. Complete growth medium (5 mL) was added to aspirate cells by pipetting and centrifuged in a sterile centrifuge tube at 200 rpm for 5 min. After that, counted viability of the cells by trypan blue staining. (23)

After trypsinization, diluted a lot of MCF-7 cells to 50000 cells/mL by using complete media, then prepared a 96 clear sterile cell culture plate and added 100 µl of the 50,000 cells/mL solution into each well; this gave 5000 cells/well for 72hrs incubation and incubated overnight in the incubator (37°C – 5% CO_2) as shown in figure 2.

On the dosing day, prepare a second well plate to transfer 5µl compounds (3a-d & 4a-d) to 125µl of warm-up media of each well plate by a multichannel. To each well of cells, added 25 µl of compounds with media from the second plate for MCF-7 cell plates and incubated overnight. On the next day, removed the plate from the incubator and placed it in the safety cabinet. The MTT solution (30 µl) added to each well, then mixed the plate in the thermo mixer for 2 minutes at 500 rpm at 37°C. Incubated for 4h at 37°C in culture hood. Finally, read absorbance at 500-600 nm.

3. RESULT AND DISCUSSION

3.1 Structure Design

Based on the general structure of raloxifene, we used benzothiophene (BT) as a building block to design our novel compounds (3a-d & 4a-d). In terms of topology-based scaffold hoping, the basic amino warhead group of raloxifene was replaced by 2-azetidinone or thiazolidinone attaching to the different substituted phenyl rings. Taken together, this would retain the polyaromatic phenolic core in the designed compounds.

The position of the warhead group on BT core switched from C3 in raloxifene to the C2 site in (3a-d & 4a-d) compounds. Ethylene diamine spacer has used to accommodate the proper direction of the warhead. The C-C linker in raloxifene was replaced by a ketone hinge. Finally, the C3 position of compounds (3a-d & 4a-d) was substituted with lipophilic chlorine atoms. (Figure 1)

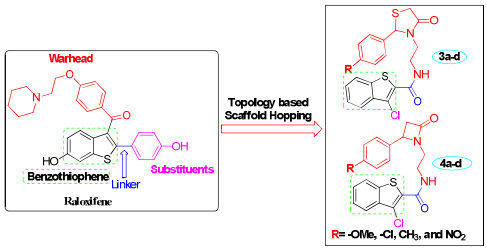
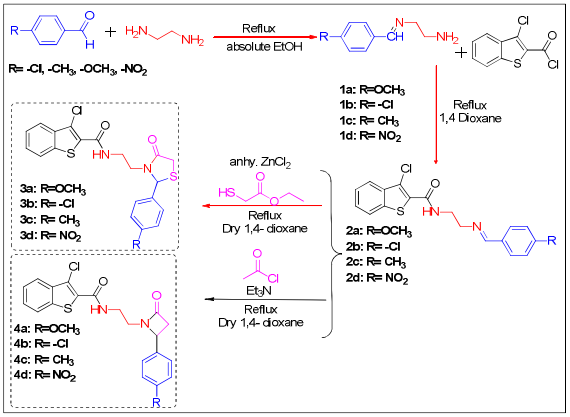


Fig. 2: Designing of novel benzothiophene using raloxifene as a model

3.2 Chemistry

The general method specified to synthesize the target compounds (3a-d & 4a-d), in Scheme 1.



Scheme 1: General synthetic pathway of the intermediates and target compounds (3a-d) & (4a-d)

Before the cyclocondensation reaction was investigated to make our final compounds (3a-d & 4a-d). Schiff base and then amide functional group had to be synthesized. We investigated the synthesis of Schiff base (C=N) by condensing the ethylene diamine with benzaldehyde derivatives to form 1a-d via hemiaminal intermediate. FT-IR, ^{1}H-NMR and ^{13}C-NMR spectra confirmed the reactions success. The FT-IR spectrum of 1a-d showed the disappearance of the absorption band of C=O at 1685 cm^{-1} and appeared a clear band assigned for C=N vibration at (1620-1597) cm^{-1}. [24, 25] This could argue as evidence of imine formation. The IR spectrums also showed a vibrational band for NH_2 and aliphatic C-H stretching. This could consider as an additional evidence of coupling ethylene diamine with benzaldehyde derivatives to form (1a-d). In terms of ^{1}H-NMR spectra of compounds (1a-d), it has been shown that the benzylic proton of Schiff base (H-C=N) appeared as a singlet at (8.66-8.33) ppm, which is in agreement with another publication[26] and the absence of generally acknowledged benzaldehyde proton at (10.2) ppm.[27] The spectra correctly reported the aromatic proton and NH_2 at (3.86-3.31) ppm. The relatively aliphatic protons ($-CH_2-CH_2-$) showed up as a singlet at (3.98-3.29) ppm. Regarding ^{13}C-NMR spectra, they stated that C=N appeared at (161.17- 162.17) ppm and the disappearance of the aldehydic C=O, which was going along with what was published.[28] These results are consistent with FT-IR results, hence the successful synthesis of (1a-d).

If we now turn to the compounds (2a-d), these compounds were characterized using FT-IR, ^{1}H-NMR and ^{13}C-NMR spectra. The amide synthesis explored via nucleophilic attack of 1° amine of (1a-d), followed by deprotonation and ended up with expelling chlorine –the leaving group (pKa < 0) – to form the 2° amide. In terms of FT-IR, there was the disappearance of the absorption band of NH_2 at (3441- 3200) cm^{-1} and appeared the clear vibrational bands such as those that were assigned for N-H symmetric stretching at (3286-3282) cm^{-1} and C=O vibrational band at (1654-1620) cm^{-1}, which is in agreement with other studies.[29, 30] Regarding ^{1}H-NMR spectra of compounds (2a-d), it has appeared the amide N-H as a singlet peak, which was overlapped with the benzylic C-H at (8.54-8.51) ppm. In addition, an extra aromatic signal representing (4 Hs) of benzothiophene was located appropriately at (7.61–7.54) ppm. At the same time, the NH_2 peak disappeared, which indicates the amide functional group was successfully formed in a mild condition. In terms of ^{13}C-NMR data, it has shown a significant feature peak that belongs to C=O at (160.33-162.52) ppm. The results of these investigations revealed that the compounds (2a-d) were synthesized and characterized successfully and in agreement with other publications.[14, 31]

Having discussed the characterization data of (1a-d & 2a-d), this section will address the cycloaddition reaction to form compounds (3a-d) with their analytic interpretation. 4-thiazolidinone was constructed by the cycloaddition reaction between Schiff base and ethyl thioglycolate in the presence of a pinch of anhydrous zinc chloride as a reducing agent.[31] The FT-IR spectrum of compounds (3a-d) showed an appearance of the absorption band of C=O at (1624-1620) cm^{-1} as reported relatively in previous publications.[32] Likely, the FT-IR spectra showed an absence of a C=N peak, which could be recognized from the sharpness of the C=O peak, which is attributed to the dual effect of two amide functional groups in the compounds (3a-d). Furthermore, C-S stretching located in the range (879-860) cm^{-1}, and C-N is stretching at (1360-1300) cm^{-1} and as reported previously.[33] Regarding ^{1}H-NMR spectra of compounds (3a-d) have been illustrated all the protons at their perspective positions. The (CH_2) characteristic signal for the proper synthesis of thiazolidinone was shown up at (3.56-3.32) ppm, which is in coinciding with another research.[29] The another evidence of the synthesis of 4-thiazolidinone comes from ^{13}C-NMR spectra. It newly formed C-S bond has been shown at chemical shift (37.33-33.01) ppm and the disappearance of the C=N chemical shift. From the aforementioned analysis, we can conclude that the 4-thiazolidinone ring was synthesized successfully, and these final compounds (3a-d) were sent to evaluate their pharmacological activity.

In the final part of the chemical synthesis, compounds (4a-d) were synthesized, and FT-IR, ^{1}H-NMR, and ^{13}C-NMR spectra were used to follow up and characterise the compounds. First of all, schiff bases (2a-d) were cyclocondesed intermolecularly with acetyl chloride to afford β-lactam (2-azetidinones) in [2+2] cycloaddition reaction (i.e. Staudinger reaction) using triethyl amine as a base.[14,

[34] The FT-IR showed that all the characteristic functional groups were in their proper position. For example, the (N-H) peak was at (3295-3286) cm^{-1}, and C=O of amide was at (1628-1620) cm^{-1}at the same time, there was no C=N peak. The ^{1}H-NMR spectra of compounds (4a-d) have illustrated all the protons at their perspective positions. The (CH_2) characteristic signal for the proper synthesis of 2-azetidinone was shown up at (3.57-3.54) ppm, which is in coincidence with other research.[35, 36] Moreover, the Ar-CH signal appeared in the range (8.52-8.51) ppm which was in agreement with Singh and Kumar (2015) studies at (8.43-8.41) ppm.[31] In ^{13}C-NMR spectra of 2-azetidinone, the characteristic signals of 2-azetidinone (CO cyclic, CH-N and CH_2) appeared in the range (174.83-169.55) ppm, (69.50-59.45) ppm and (47.23- 37.93) ppm, respectively. The spectral data provide support to the proposed structures to all the synthesized compounds, and these final compounds (4a-d) were sent to evaluate their pharmacological activity.

3.3. Pharmacological Evaluation

The newly synthesized compounds (3a-d & 4a-d) were screened for in vitro anti-breast cancer activity using the MCF-7 cell line. We explored MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay to evaluate the cell viability, proliferation and cytotoxicity and employed tamoxifen (TAM) as a standard reference. The compounds (3a-d & 4a-d) were studied in terms of the time response and dose response curves and comparison with TAM.

In the case of the time response curve, the freshly cultivated MCF-7 cells were treated with (25 µM) of compounds (3a-d & 4a-d) and control (TAM) at different times. The response was measured at 24 hr, 48 hr and 72 hr. It has been described in Figure 3, the response is directly proportional to time, which reached the maximum cell death per cent after 72 hr, (Fig. 3).

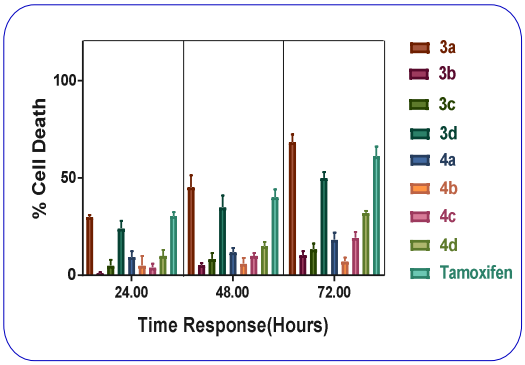


Fig. 3: In vitro % cell death of the breast cancer (MCF-7) cells was detected by MTT assay. The results of MCF7 cells post 24, 8 and 72h treatment of 25 µM of all target compounds with Tamoxifen as control. A microplate reader measured the absorbance was at 540 nm (reference wavelength 650 nm). The results represent the mean absorbance ± SEM of 3 independent experiments using Prism Pad 8.1 software to drawbars

On the other hand, the dose-response curvatures generated by Prism Pad 8.1 using nonlinear regression analysis of compounds in MCF-7 cells are shown below. The IC_{50} values were obtained for a range of concentrations of compounds from (1.56 – 200 µM) by MTT assay. All compounds were tested using the same cell line (MCF-7). As shown in (Fig. 4), compound (3a) and (3d) were highly potent in inhibiting MCF-7 cells than TAM. Furthermore, as seen from (Fig. 4), compound (4d) was slightly less potent than TAM on the MCF-7 cell line using the MTT assay. Whereas, other compounds such as [3b, 3c, & 4a-c] are less efficient towards MCF-7, (Fig. 4)

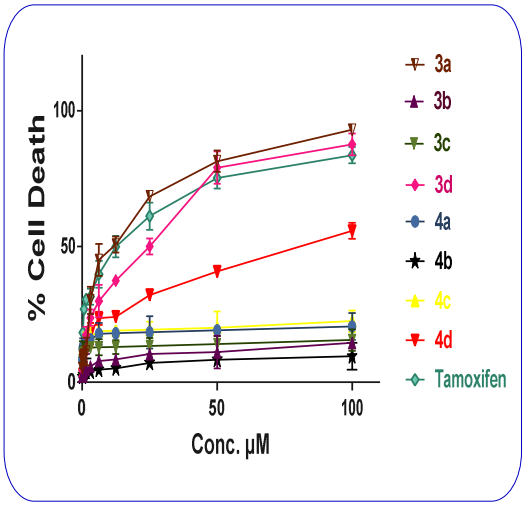


Fig. 4: The dose-response curve of all compounds in the breast cancer (MCF-7) cells was detected by MTT assay. The results of MCF7 cells post 72h treatment of 0.05, 0.15, 0.32, 0.75, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150 and 200 µM of (3a-d) & (4a-d) compared with tamoxifen as control. The absorbance was measured at 540 nm (reference wavelength 650 nm) using a microplate reader. The results represent the mean absorbance ± SEM of 3 independent experiments using Prism Pad 8.1 software to draw bars

In terms of IC_{50} at the MCF-7 cell line, it has been shown that both (3a) and (3d) exhibited excellent and higher IC_{50} values (10.32µM and 11.35µM, respectively) than TAM (18.02µM). Moreover, (4d) has shown a moderately lower IC_{50} value of (22.65µM) than the control (TAM) (Fig. 5). However, the rest of the derivatives [3b, 3c, & 4a-c] were less active than TAM, with IC_{50} values with IC_{50} above than 100 µM, (Table 1).

Table 1

IC_{50} for target compounds Vs Tamoxifen at 72h

Compounds	IC_{50} (µM)
3a	10.32
3b	147
3c	132
3d	11.35
4a	125
4b	178
4c	116
4d	22.65
Tamoxifen	18.02

The analysis showed significant results of benzo[*b*]thiophene derivatives (in particular: 3a, 3d, & 4d) in comparison with the reference (Tamoxifen). These compounds could be considered as a lead candidate to fight breast cancer, (Fig. 5).

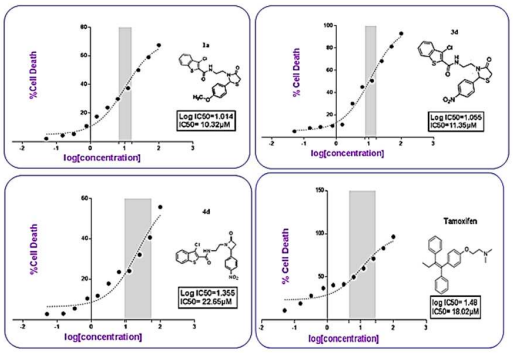


Fig. 5: Dose-response curves of IC_{50} for 3a, 3d, 4d and tamoxifen (Control). MCF-7 cells were treated for 72h with 0.05, 0.15, 0.32, 0.75, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150 and 200 µM dose ranges of tamoxifen. The dose-response for tamoxifen was plotted over log- transformed tamoxifen concentrations. IC_{50} values were determined using nonlinear regression analysis (Prism Pad 8.1). The results represent the standard error of the mean (SEM) for triplicate data

4. CONCLUSION

In this study, new BT derivatives have been designed and synthesized starting from the BT scaffold. The structures of all new compounds were proved using FT-IR, ^{1}H-NMR, and ^{13}C-NMR spectra. The compounds were evaluated for their anti-proliferative activity by MTT assay and using an MCF-7 cell line and tamoxifen as a reference compound. Thus, it can be concluded that both compounds (3a) and (3d) have the potential to be developed as an anticancer agent.

5. ACKNOWLEDGEMENT

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6. CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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