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ARTICLE

Synthesis, Pharmacological Evaluation, and *In-Silico* Studies of Thiophene Derivatives

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ABSTRACT

The relevance of Retinoic acid receptor-related orphan receptors in cancer progression has sparked interest in developing multifunctional therapeutics. In the search for potentially active novel compounds with anticancer characteristics, the Gewald reaction was employed to develop different thiophene derivatives **(8a–8i)**. Physico-chemical and spectroanalytical investigations verified the molecular structures of the synthesized derivatives. Using an *in vitro* primary anticancer assay, NCI chose all of the synthesized molecules as prototypes and assessed their anticancer efficacy against a panel of various cancer cell lines representing nine distinct neoplasms. The compounds were found to have a wide range of anticancer activity. Following significant anticancer efficacy against all cell lines in the initial screening, compound **8e** was chosen for a five-dose test. Compound **8e** inhibited growth at concentrations ranging from 0.411 to 2.8 μM. The antioxidant activity of the compounds was further evaluated using the radical scavenging action of the stable DPPH free radical. In comparison to Ascorbic Acid, compounds **8e** and **8i** showed outstanding antioxidant activity, while the remaining compounds in the series demonstrated acceptable antioxidant activity. In a molecular docking investigation, **8e** demonstrated excellent docking scores inside the binding pocket of the specified pdb-id (6q7a), complementing the results of anticancer screening. Based on our results, novel ethyl 5-acetyl-2-amino-4-methylthiophene-3-carboxylate derivatives could be useful in the development of potential anticancer treatments.

KEYWORDS

Thiophene; anticancer; antioxidant; RORγt inhibitors; SAR; *in-silico* studies

Abbreviations

DPPH:	2,2-diphenyl-2-picrylhydrazyl
GI_{50}:	Growth inhibition of 50% cell
LC_{50}:	Lethal concentration that gives 50% cell kill
MG_MID:	Full panel mean-graph midpoint
NCI:	National Cancer Institute
ppm:	Parts per million
RORs:	Retinoic acid receptor-related orphan receptors

ROS: Reactive oxygen species
SAR: Structure activity relationship
SRB: Sulforhodamine B protein assay
TGI: Total Growth Inhibition
TMS: Tetramethylsilane

1 Introduction

In the modern medical field, cancer is the most prevalent, particularly complex, and deadly illness [1]. It has been one of the world's main sources of mortality over the last decade. According to the World Health Organization, an estimated 9.6 million people died of cancer in 2018 [2,3]. To provide better and more successful cancer therapy and cure, the medical scientific community confronts a significant challenge [4–6]. This task involves the development of new medications, therapies, and care for cancer patients. These neoplastic tumor cells are varied and diversified, with the ability to proliferate rapidly. These malignant neoplasms may infiltrate or spread to other parts of the body through the blood circulation and lymphatic networks [7–9].

Chemotherapeutic drugs are one treatment option for different types of cancer. However, these medicines are associated with many drawbacks, including drug tolerance, systemic cytotoxicity, and a narrow therapeutic efficacy [10–13]. Novel chemotherapeutic drugs with well-established mechanisms must be developed to overcome these limitations. The identification of cytotoxic molecules, or agents that may kill cancer cells, has led to advances in anticancer treatment. These medicines improve cancer patients' survival and well-being [14,15]. Numerous compounds having heterocyclic rings have already shown significant antiproliferative activity, especially those containing thiophene rings. Thiophene derivatives stand out among biomolecules employed in research to evaluate biological activity due to their diverse properties [16].

To improve specificity and safety profiles, several synthetic routes are being used to develop various novel thiophene derivatives [17]. 2-amino-thiophenes have earned a significant interest among the thiophene compounds. A great deal of attention has been paid to 2-amino-thiophenes recently, owing to improvements in their synthetic methods, stability, availability, and structural simplicity, which makes them an important moiety in pharmaceuticals [18,19]. Furthermore, antifungal [20], antibacterial [21,22], antileishmanial [23], anxiolytic [24], anti-inflammatory [25], antiplatelet [26], antioxidant [27], antiandrogenic [28], and anti-diabetes [29] actions have been revealed for thiophene and its analogues.

A key element in the growth of cancer and as potential therapeutic targets for different malignancies has been detected with nuclear receptors. Retinoic acid receptor-related orphan receptors (RORs) are a subfamily of the thyroid hormone receptor and belong to the orphan nuclear receptor family, which is a nuclear receptor subfamily. RORα, RORβ, and RORγ are members of the ROR subfamily. These receptors are involved in the development of autoimmune diseases, inflammatory diseases, circadian rhythm, secondary lymphoid tissue, and homeostasis-metabolism by triggering transcription through ligand-dependent interactions with co-regulators [30].

According to recent studies, RORγ and its isoforms are expressed in the thymus and lymphoid organs, but also tend to be involved in some cancers, including lymphoma, melanoma, lung, and ovarian cancer [31].

RORγt is an immune cell-specific isoform of ROR with a ligand-binding domain receptor. Cellular differentiation and activation of TH-17 cells result in the production of pro-inflammatory mediators such

as interleukin-17 (IL-17), which itself is linked to malignancies such as lung, colon, and ovarian cancer [32–35]. As a result of this, researchers are always looking for new molecules that have the potential to block RORγt and use them to treat cancer-related conditions. Ursolic acid, digoxin, azole-type fungicides, and SR211 are all known RORγt receptor inhibitors.

The approach for designing the compound was based on heterocyclic structures previously reported as RORγt inhibitors (Fig. 1) [36].

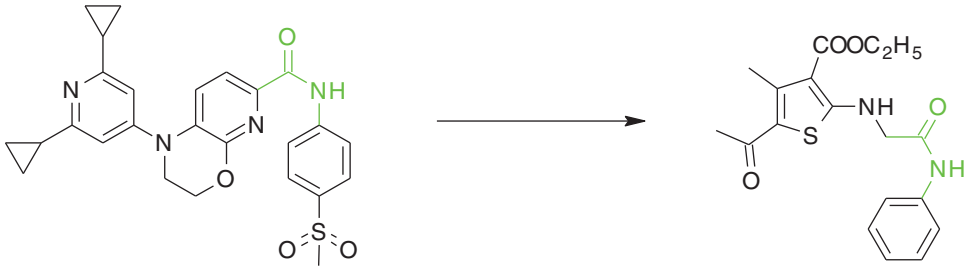


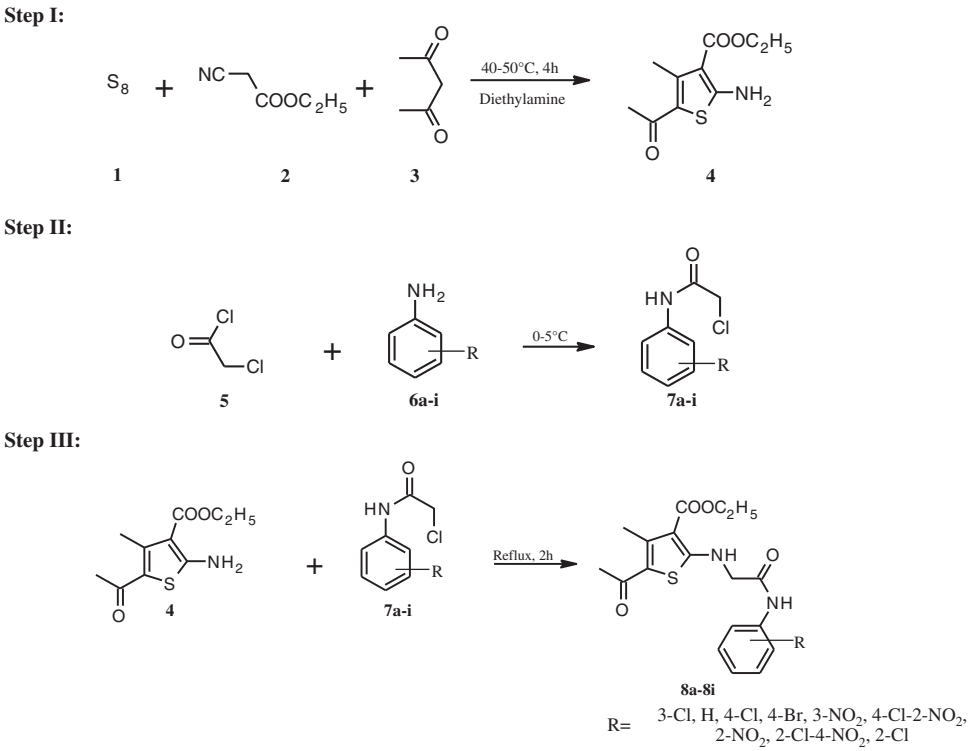
Figure 1: Design of target compounds

Inflammatory disorders, cancer, rheumatoid arthritis and aging are among the pathological situations where reactive oxygen species (ROS) are involved. Oxidative stress is linked with an increase in free radical consumption or a decrease in antioxidant concentration, which affects cell membranes and other components such as DNA, lipids, proteins, and lipoproteins [37]. Excess hydroxyl radicals and peroxynitrite, for example, may induce lipid peroxidation by causing damage to cell membranes and lipoproteins. Malondialdehyde and conjugated diene compounds are produced during this process, both of which are cytotoxic and mutagenic.

Experimental investigations on animals, as well as *in vitro* studies, have indicated that ROS is an important factor in carcinogenesis and that there is a critical balance between free radical production and antioxidant defense as a preventative force against cancer. However, the exact mechanism through which antioxidants exert their anti-carcinogenic actions is still unclear. Based on the findings so far, it seems that the most probable mechanism for antioxidants anticancer action is by [38–40]:

1. possibility of reducing the production of “activated” carcinogenic species through Phase I biotransformation enzymes,
2. scavenging free radicals, ROS, and electrophiles; and
3. enhancement of electrophile detoxification *via* induction of Phase II detoxification enzymes such as glutathione S-transferases and NAD(P)H: quinone reductase.

ROS is known to induce a variety of human malignancies, and given the severity of the disease, the anticancer profile of thiophene-based compounds has mostly been thoroughly studied [41–46]. The present research was carried out as an outcome of this finding to design strong RORγt inhibitors via the introduction of various substituted aromatic moieties at the thiophene terminal (Scheme 1) and assess their therapeutic potential to continue our search for novel anticancer agents.



Scheme 1: Synthetic pathway for compounds 8a-i

2 Experimental Section

2.1 Chemistry

2.1.1 Chemicals and Instrumentations

Preliminary material required for experimental work was procured from the authorized suppliers and used without further purification. For tracking the reaction progress, TLC was carried out using glass plates coated with silica gel G using mobile phase {ethyl acetate/n-hexane (1:2) and ethyl acetate/benzene (1:1)}. The plates were visualized in an iodine chamber. Open capillary melting point apparatus was used to determine melting points and reported as uncorrected. IR spectra (in KBr) were acquired using the DRS 8000A accessory technique on a Shimadzu IR Affinity-1 FTIR spectrophotometer. Both ^{1}H and ^{13}C NMR spectral analysis of synthesized compounds in $CDCl_3$ with tetramethylsilane (TMS) as an internal standard were recorded on Bruker Avance-II 400 NMR Spectrometer operating at 500 MHz. Chemical shift (δ) values were reported in parts per million (ppm). Using Waters Q-TOF (ESI–MS) micromass, mass spectra were recorded. SAIF, Panjab University, Chandigarh conducted spectral analysis, including mass spectroscopy and NMR studies.

2.1.2 Step 1: Synthesis of ethyl 5-acetyl-2-amino-4-methylthiophene-3-carboxylate (4)

At room temperature, sulphur (0.06 mol) was added with stirring to an equimolar (0.05 mol) mixture of ethyl cyanoacetate and acetylacetone. Diethylamine (0.05 mol) was added dropwise to this heterogeneous mixture. The reaction mixture was agitated for 4 h at a temperature of 40–50°C. TLC was used to monitor the reaction's progress, and the mixture was left at room temperature overnight. After filtering, washing with water, drying, and recrystallization from ethanol, the brown precipitate was obtained.

Compound 4: Yield: 34%; M.P.: 150–152°C; $R_f = 0.66$; IR (KBr, cm^{-1}): 785 (C-S-C str.), 1257 (C-O-C str.), 1583 (C=C str.), 1666 (C=O str.), 2968 (C-H str.), 3408 (N-H str.)

2.1.3 Step 2: Synthesis of N-substituted α-chloro Acetanilides (7a-i)

In a saturated sodium acetate solution (25 mL), a suitable substituted aromatic amine (0.05 mol) was dissolved. If the substance is not completely dissolved, the mixture is warmed up until it is dissolved. It was subsequently cooled in an ice bath with stirring. To this reaction mixture, chloroacetyl chloride (0.07 mol) was added dropwise to avert a vigorous reaction. For 5–6 h, the mixture was kept at room temperature. The product obtained was then filtered, washed with distilled cold water, dried, and recrystallized from aqueous ethanol. Physicochemical characteristics and spectral analysis data of *N*-substituted α-chloro acetanilides are included in Table 1.

Table 1: Physicochemical characteristics and spectral analysis data of *N*- substituted α-chloro acetanilides

Compound	Mol. Formula	Mol. Weight	Color	% Yield	R_f Value*	Melting Point**	IR (KBr, cm^{-1})
7a	$C_8H_7Cl_2NO$	204	White	24.53	0.67	112–114	1548(C=C str. in ring), 1678 (C=O str.), 3269(N-H str.), 3116 (C-H str. aromatic), 770(C-Cl str. aromatic).
7b	C_8H_8ClNO	169	White	86.35	0.71	110–112	1556(C=C str. in ring), 1672 (C=O str.), 3267(N-H str.), 3099–3145(C-H str. aromatic), 750(C-Cl str.).
7c	$C_8H_7Cl_2NO$	204	White	76.07	0.74	108–110	1556(C=C str. in ring), 1666 (C=O str.), 3263(N-H str.), 3082–3130(C-H str. aromatic), 777(C-Cl str. aromatic).
7d	$C_8H_7BrClNO$	248	Light Brown	76.04	0.72	118–122	1552(C=C str. in ring), 1672 (C=O str.), 3265(N-H str.), 3126 (C-H str. aromatic), 499(C-Br str. aromatic), 780(C-Cl str.).
7e	$C_8H_7ClN_2O_3$	214	Brown	40.18	0.71	106–110	1531(C=C str. in ring), 1681 (C=O str.), 3086(C-H str. aromatic), 3304(N-H str.), 1350 (C-NO_2 str. aromatic).
7f	$C_8H_6Cl_2N_2O_3$	249	Orange	48.63	0.6	124–126	3093 (C-H str. aromatic), 1342 (C-NO_2 str. aromatic), 1504 (C=C str. in ring), 1687 (C=O str.), 3354 (N-H str.), 770 (C-Cl str. aromatic).
7g	$C_8H_7ClN_2O_3$	214	Creamy White	10.90	0.68	104–106	1517 (C=C str. in ring), 1693 (C=O str.), 3018 (C-H str. aromatic), 3307 (N-H str.), 1338 (C-NO_2 str. aromatic).
7h	$C_8H_6Cl_2N_2O_3$	249	Yellow	43.79	0.67	104–106	1321 (C-NO_2 str. aromatic), 1502 (C=C str. in ring), 1693

(Continued)

Table 1 (continued)

Compound	Mol. Formula	Mol. Weight	Color	% Yield	R_f Value*	Melting Point**	IR (KBr, cm^{-1})
							(C=O str.), 3373 (N-H str.), 750 (C-Cl str. aromatic).
7i	$C_8H_7Cl_2NO$	204	White	23.82	0.7	118–120	3043 (C-H str. aromatic), 1531 (C=C str. in ring), 1672 (C=O str.), 758 (C-Cl str. aromatic), 3267 (N-H str.).

Note: * R_f value (Solvent System: Ethyl acetate: Benzene (1:1)).
** Melting point in °C.

2.1.4 Step 3: Synthesis of Ethyl 5-acetyl-2-({2- [(substitutedphenyl)amino]-2-oxoethyl}amino)-4-methylthiophene-3- carboxylate (8a-8i)

In equimolar proportions (0.05 mol), various N-substituted -chloro acetanilides **($7A_1$–A_9)** and compound **4** were mixed in 1,4-dioxane (15 mL). Following the addition of triethylamine solution (0.005 mol), the reaction mixture was refluxed for 2 h. The reaction mixture was poured over crushed ice after cooling. The resulting product was filtered and dried after being washed with potassium bicarbonate (1%). The reaction was monitored and R_f values for 8a-8i were determined using the ethyl acetate/n-hexane solvent system (1:2).

***Compound 8a: Ethyl 5-acetyl-2-({2-[(3-chlorophenyl)amino]-2-oxoethyl}amino)-4-methylthiophene-3-carboxylate*:** Brown Solid (77.7%); M.P.: 108–110°C; R_f = 0.7; IR (KBr, cm^{-1}): 3296 (N-H str.), 3408 (N-H str. coupled), 1603 (C=C str.), 3087 (Ar-H str.), 877 (C-C str.), 1664 (C=O str.), 1477 (C-N str.), 819 (C-Cl str.), 785 (C-S str.); ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.49 (s, 2H, CH_2), 8.19 (s, 1H, -CONH), 7.13-7.47 (m, 4H, Ar-H), 4.38 (q, 2H, OCH_2CH_3), 4.20 (s, 1H, NH), 1.42 (t, 3H, OCH_2CH_3), 2.55 (s, 3H, $-COCH_3$), 2.19 (s, 3H, $-CH_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 182.8, 167.7, 166.3, 164.0, 149.4, 139.5, 134.3, 129.9, 124.8, 120.4, 120.3, 109.1, 109.1, 61.5, 47.8, 27.2, 15.1, 14.6; ESI-MS (m/z): 396.37 (M+1).

***Compound 8b: Ethyl 5-acetyl-4-methyl-2-{[2-oxo-2-(phenylamino)ethyl] amino}thiophene-3-carboxylate*:** Creamy solid (73.33%); M.P.: 130–132°C; R_f = 0.66; IR (KBr, cm^{-1}): 786 (C-S str.), 3297 (N-H str.), 3405 (N-H str. coupled), 1663 (C=O str.), 3097 (Ar-H str.), 1499 (C-N str.), 1618 (C=C str.), 859 (C-C str.), 1284 (C-O str.), 691 (Monosubsti. Ring); ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.47 (s, 2H, CH_2), 7.50–7.14 (m, 5H, Ar-H), 4.30 (q, 2H, OCH_2CH_3), 4.40 (s, 1H, NH), 2.57 (s, 3H, $-COCH_3$), 1.40 (t, 3H, OCH_2CH_3), 2.43 (s, 3H, $-CH_3$), 8.46 (s, 1H, -CONH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.2, 167.7, 166.3, 164.0, 149.4, 137.7, 129.3, 126.4, 123.7, 121.4, 109.1, 86.3, 61.5, 47.8, 27.2, 15.1, 16.87, 14.6; ESI-MS (m/z): 361.61 (M+1).

***Compound 8c: Ethyl 5-acetyl-2-({2-[(4-chlorophenyl)amino]-2-oxoethyl}amino)-4-methylthiophene-3-carboxylate*:** Buff colored solid (92%); M.P.: 122–124°C; R_f = 0.67; IR (KBr, cm^{-1}): 778 (C-S str.), 826 (C-Cl str.), 863 (C-C str.), 1275 (C-O str.), 1474 (C-N str.), 1606 (C=C str.), 1665 (C=O str.), 3408 (N-H str. coupled), 3085 (Ar-H str.), 3297 (N-H str.); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.28–7.48 (m, 4H, Ar-H), 8.43 (s, 1H, -CONH), 4.48 (s, 2H, CH_2), 4.33 (q, 2H, OCH_2CH_3), 4.24 (s, 1H, NH), 1.39 (t, 3H, OCH_2CH_3), 2.55 (s, 3H, $-COCH_3$), 2.43 (s, 3H, $-CH_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.2, 181.7, 167.7, 166.3, 164.1, 137.79, 149.4, 136.5, 129.2, 128.6, 126.4, 122.2, 109.1, 61.5, 47.8, 27.2, 15.1, 14.6; ESI-MS (m/z): 396.16 (M+1).

***Compound 8d: Ethyl 5-acetyl-2-({2-[(4-bromophenyl)amino]-2-oxoethyl}amino)-4-methylthiophene-3-carboxylate*:** Light Yellow (93%); M.P.: 134–136°C; R_f = 0.6; IR (KBr, cm^{-1}): 3300 (N-H str.), 3410 (N-

H str. coupled), 1606 (C=C str.), 862 (C-C str.), 3081 (Ar-H str.), 1663 (C=O str.), , 657 (C-S str.), 1488 (C-N str.), 1250 (C-O str.), 773 (C-Br str. coupled); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.24 (s, 1H, -CONH), 4.36 (s, 2H, CH_2), 4.34 (q, 2H, OCH_2CH_3), 7.26-7.64 (m, 4H, Ar-H), 4.42 (s, 1H, NH), 2.15 (s, 3H, -$COCH_3$), 2.56 (s, 3H, -CH_3), 1.39 (t, 3H, OCH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.2, 167.7, 166.3, 164.0, 149.4, 136.2, 131.7, 136.2, 131.7, 126.4, 122.1, 118.2, 109.1, 61.5, 47.8, 27.2, 15.1, 14.6; ESI-MS (m/z): 440.61 (M+1), 441.41 (M+2).

***Compound 8e: Ethyl 5-acetyl-2-({2-[(3-nitrophenyl)amino]-2-oxoethyl}amino)-4-methylthiophene-3-carboxylate*:** Light Brown solid (67%); M.P.: 122–124°C; R_f = 0.66; IR (KBr, cm^{-1}): 2986 (Ar-H str.), 1310 (C-NO_2 str. aromatic), 3415 (N-H str. coupled), 3295 (N-H str.), 786 (C-S str.), 1684 (C=O str.), 1476 (C-N str.), 1526 (N–O asymmetric stretch), 1273 (C-O str.), 835 (C-C str.); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.2704 (s, 1H, -CONH), 7.56–7.89 (m, 4H, Ar-H), 4.54 (s, 2H, CH_2), 4.34 (q, 2H, OCH_2CH_3), 1.39 (t, 3H, OCH_2CH_3), 3.96 (s, 1H, NH), 2.55 (s, 3H, -$COCH_3$), 2.44 (s, 3H, -CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 183.5, 167.7, 166.2, 164.1, 149.4, 148.7, 139.6, 129.2, 127.0, 126.4, 120.2, 115.8, 109.2, 61.5, 47.8, 27.2, 15.1, 14.3; ESI-MS (m/z): 406.24 (M+1).

***Compound 8f: Ethyl 5-acetyl-2-({2-[(4-chloro-2-nitrophenyl)amino]-2-oxoethyl}amino)-4-methylthiophene-3-carboxylate*:** Yellowish Orange solid (94.1%); M.P.: 130–132°C; R_f = 0.66; IR (KBr, cm^{-1}): 3410 (N-H str. coupled), 3296 (N-H str.), 2873 (C-H str.), 1661 (C=O str.), 1455 (N–O asymmetric stretch), 1589 (C=C str.), 914 (C-C str.), 1254 (C-O str.), 2993 (Ar-H str.), 891 (C-Cl str.), 786 (C-S str.); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.46–7.85 (m, 3H, Ar-H), 8.84 (s, 1H, -CONH), 1.39 (t, 3H, OCH_2CH_3), 4.49 (s, 2H, CH_2), 4.35 (q, 2H, OCH_2CH_3), 4.36 (s, 1H, NH), 2.47 (s, 3H, -$COCH_3$), 2.59 (s, 3H, -CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.2, 167.7, 166.3, 164.0, 149.4, 148.7, 139.6, 129.2, 127.0, 126.4, 120.2, 115.8, 109.8, 84.6, 47.8, 27.2, 15.1, 14.7; ESI-MS (m/z): 440.51 (M+1).

***Compound 8g: Ethyl 5-acetyl-2-({2-[(2-nitrophenyl)amino]-2-oxoethyl}amino)-4-methylthiophene-3-carboxylate*:** Light Yellow solid (91.7%); M.P.: 110–112°C; R_f = 0.67; IR (KBr, cm^{-1}): 1588 (C=C str.), 2986 (Ar-H str.), 3410 (N-H str. coupled), 3295 (N-H str.), 2854 (C-H str.), 1665 (C=O str.), 1513 (N-O asymmetric stretch), 1458 (C-N str.), 1311 (C-NO_2 str. aromatic), 1271 (C-O str.), 1257 (C-N str.); 847 (C-C str.), 738 (C-S str.); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43–8.31 (m, 4H, Ar-H), 8.32 (s, 1H, -CONH), , 1.39 (t, 3H, OCH_2CH_3), 4.41 (s, 2H, CH_2), 4.34 (q, 2H, OCH_2CH_3), 4.39 (s, 1H, NH), 2.09 (s, 3H, -$COCH_3$), 2.54 (s, 3H, -CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.2, 183.5, 167.7, 164.9, 149.4, 148.7, 139.6, 129.2, 127.0, 126.4, 120.0, 115.8, 109.8, 84.6, 47.3, 27.4, 15.0, 14.9; ESI-MS (m/z): 406.1 (M+1).

***Compound 8h: Ethyl 5-acetyl-2-({2-[(2-chloro-4-nitrophenyl)amino]-2-oxoethyl}amino)-4-methylthiophene-3-carboxylate*:** Yellow solid (88.6%); M.P.: 118–120°C; R_f = 0.60; IR (KBr, cm^{-1}): 947 (C-C str.), 3376 (N-H str.), 1510 (N-O asymmetric stretch), 893 (C-Cl str.), 3496 (N-H str. coupled), 1262(C-O str.), 3117 (Ar-H str.), 1665 (C=O str.), 1588 (C=C str.), 1310 (C-NO_2 str. aromatic), 786 (C-S str.); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52–8.32 (m, 3H, Ar-H), 6.48 (s, 1H, -CONH), 4.52 (s, 2H, CH_2) , 1.39 (t, 3H, OCH_2CH_3), 4.35 (q, 2H, OCH_2CH_3), 5.99 (s, 1H, NH), 2.13 (s, 3H, -$COCH_3$), 2.57 (s, 3H, -CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.2, 183.0, 166.3, 164.0, 149.4, 148.7, 139.6, 129.2, 127.0, 126.4, 120.2, 115.8, 109.1, 61.5, 47.8, 27.2, 15.1, 14.9; ESI-MS (m/z): 440.73 (M+1).

***Compound 8i: Ethyl 5-acetyl-2-({2-[(2-chlorophenyl)amino]-2-oxoethyl}amino)-4-methylthiophene-3-carboxylate*:** Light Brown solid (75.44%); M.P.: 120–122°C; R_f = 0.72; IR (KBr, cm^{-1}): 1504 (C-N str.), 3296 (N-H str.), 3410 (N-H str. coupled), 2986 (C-H str.), 3070 (Ar-H str.), 1665 (C=O str.), , 758 (C-S str.), 1604 (C=C str.), 1256 (C-O str.), 810 (C-Cl str.), 836 (C-C str.); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07–7.39 (m, 4H, Ar-H), 5.18 (s, 1H, -CONH), 4.48 (s, 2H, CH_2), 1.40 (t, 3H, OCH_2CH_3), 4.34 (q, 2H, OCH_2CH_3), 4.40 (s, 1H, NH), 2.13 (s, 3H, -$COCH_3$), 2.55 (s, 3H, -CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.7, 183.5, 167.7, 166.3, 164.0, 149.4, 148.7, 139.6, 129.2, 127.0, 126.4, 120.2, 109.2, 61.5, 47.8, 27.4, 15.0, 14.6; ESI-MS (m/z): 396.42 (M+1).

2.2 Pharmacological Evaluation of the Synthesized Derivatives

2.2.1 Assessment of Anticancer Activity

Full NCI 60 Cell Panel Assay in Single Doses

National Cancer Institute (NCI) identified nine compounds with anticancer efficacy in the full NCI 60 human tumor cell screen program. The compounds were first evaluated on 60 cancer cell lines, including breast, CNS, colon, leukemia, melanoma, lung, ovarian, prostate, and renal cancer cell lines, at a concentration of 10^{-5} M. A mean graph of the percentage growth of treated cells showed the behavior of the chosen compounds. The percentage growth was determined using spectrophotometry in contrast to controls that were not administered the test entities. The continuous drug exposure procedure was evaluated for 48 h using a Sulforhodamine B (SRB) protein assay and cell viability was determined [47–49].

Full NCI 60 Cell Panel Assay in Five Doses

Following the significant suppression of growth by compound 8e, further study was performed at 10-fold dilutions of five doses (10^{-4} to 10^{-8} M). The dose-response parameters GI_{50}, TGI, and LC_{50} were used to evaluate the drug. GI_{50} value (Growth Inhibition of 50% of Cells) indicates a compound concentration that prevents net cell growth by 50%. TGI (Total Growth Inhibition) is the concentration of a substance that inhibits total growth and therefore indicates its cytostatic effect. LC_{50} value (Lethal concentration that kills 50% of cells) shows cytotoxicity and refers to the concentration of a compound that results in a net loss of 50% of the initial cells following a 48-hour incubation period.

The percentage growth curve is calculated as follows:

$$[(T - To)/(C - To)] \times 100 \quad (1)$$

where,

T is the cell count at day 3 at the test concentration,

T_o is the cell count at day 0, and

C is the vehicle control (without drug) cell count

The GI50 and TGI values are calculated utilizing drug concentrations that resulted in 50% and 0% growth after 48 h of drug intake, respectively.

$$\text{For } GI_{50} \text{ Value : } [(T - To)/(C - To)] \times 100 = 50 \quad (2)$$

$$\text{For TGI Value : } [(T - To)/(C - To)] \times 100 = 0 \quad (3)$$

$$\text{For } LC_{50} \text{ Value : } [(T - To)/(C - To)] \times 100 = -50 \quad (4)$$

when $T < T_o$.

The results were derived by using methods specified by the NCI/NIH Development Therapeutic Program. Following the determination of log GI_{50} values, mean-graph midpoint (MG_MID) values for the whole panel were computed. Because these numbers are presented in terms of concentration, they are more relevant for evaluating the activity. The obtained results are logarithmic concentration values showing 50% inhibition based on the test protocol. If the value is higher than –4, the compound is inactive [50].

2.2.2 Assessment of Antioxidant Activity

The Shimada method, which is based on the theory of scavenging the 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical, was used to evaluate the free radical scavenging activities of synthesized compounds in reference to ascorbic acid [51]. Different concentrations of synthesized derivatives and ascorbic acid (10–100 μg/ml) were prepared in methanol, and 1 mL of each concentration of test compound and ascorbic

acid was added to 1 mL of 0.1 mM DPPH solution. The absorbance was assessed using UV at 517 nm once the mixture was held in a dark place at room temperature for 30 minutes after intense shaking [52].

The following formula was used to calculate the % scavenging of the free radical DPPH.

$$\%\text{scavanging} = \frac{\text{Absorbance of control} - \text{Absorbance of test compouds/Std.}}{\text{Absorbance of control}} \times 100 \quad (5)$$

A graph depicting percent inhibition against various concentrations of synthesized derivatives was used to calculate the IC_{50} value for each sample. The IC_{50} value is the test sample concentration that causes a 50% reduction in the initial DPPH concentration. A higher free radical scavenging activity is indicated by a lower mean concentration value of the inhibitor.

2.3 In silico Studies

For molecular docking studies, the preferred target protein (PDB id-6q7a) was retrieved from the RCSB Protein data bank [53]. Protein Preparation Wizard was used to prepare the chosen PDB file for the molecular docking study. By incorporating polar hydrogen bonds, removing redundant water molecules, and then adding and distributing charge, the receptor molecule was optimized. With the help of the AutoDock software, the processed receptor molecule was saved in *.pdbqt format. The amino acids Phe388 and His479 aided in the active binding of ligand to the RORγt receptor. A grid around the co-crystallized ligand is created, allowing it to be eliminated and new compounds to be bound to the same active site to analyze their receptor interactions. The overlay method and chemical resemblance were used to verify the molecular docking process when docking a specific ligand with a relevant macromolecule. The docking score obtained using molecular docking software was used to examine the results. The docking score was designated by a negative value. Lower the docking score, the greater the ligand's affinity for the receptor [54].

3 Result and Discussion

3.1 Chemistry

In this study, nine new thiophene derivatives (8a–8i) were synthesized using Gewald synthesis. Intermediate (4) was synthesized by reacting ethyl cyanoacetate, acetylacetone, and sulfur with diethylamine as a base. Under cold conditions, aromatic amines and chloroacetyl chloride were reacted to give N-substituted alpha-chloroacetanilides (7a-i). Target compounds were produced in 75–86% yields in the presence of triethylamine by reacting equimolar quantities of intermediate and various *N*-substituted chloroacetanilides. The synthesized derivatives were characterized using a variety of spectroscopy approaches, all of which were consistent with the assigned chemical structures.

3.2 Pharmacological Evaluation of the Synthesized Derivatives

3.2.1 Anticancer Activity

The NCI, Bethesda, USA, investigated the anticancer activity of the selected nine compounds (8a–8i) on 60 human cancer cell lines. Table 2 includes the behavior of the identified compounds as a mean graph of the percent growth of treated cells. The highest mean activity was observed to be –16.98% for 8e when the percentage of growth inhibition of substance-treated tumor cells (10^{-5} M concentration of compounds) was compared to non-treated tumor cells. Some cell lines were, in particular, more sensitive to the compounds examined and major individual results were obtained. When contrasting the percentage of growth inhibition of substance-treated tumor cells to those that were not treated, as seen in Table 3, it was established that lung cancer, renal cancer, leukemia, CNS, and breast cancer were the most vulnerable cancer cell lines. Compound 8e was investigated further with a 5-log dose molar range after inhibiting cell growth at 10^{-5} M concentration in a variety of cell lines. The findings are described in Table 4 in terms of GI_{50}, TGI, and LC_{50} values. 8e reported GI_{50} values ranging from 0.411 to 2.8 μM. TGI values

>100 μM were seen in all leukemia cancer cell lines for the specified compound. The MG_MID values in Table 5 were estimated after calculating their log GI_{50} values in reference to Melphalan. These values are more relevant for assessing the activity as they are revealed in terms of concentration. The compound is inactive if the value exceeds –4. The substantial activity was seen in compound 8e with a MG_MID value of –5.82. These concentration values were comparable to the percentage growth values stated earlier. It was also selective against all cancer cell lines, with logGI_{50} values varying from –5.67 to –5.92. Those obtained concentration values corresponded to the previously indicated percentage growth values.

Table 2: Results of anti-cancer screening as growth percent at single dose (10^{-5} M) assay

Compound	NSC Code (provided from NCI)	BC	CNSC	CC	L	M	NSCLC	OC	PC	RC	Mean Growth Percent
8a	D-827790/1	100.07	101.62	104.14	105.72	105.17	97.12	108.03	109.38	101.24	102.99
8b	D-827787/1	96.45	96.90	103.23	107.17	106.32	95.26	107.95	101.97	96.91	101.25
8c	D-827788/1	98.75	99.03	106.51	107.83	104.20	95.43	109.69	105.77	103.30	103.03
8d	D-827789/1	102.96	101.92	107.13	108.22	105.22	98.46	115.09	107.16	102.23	105.00
8e	D-827791/1	–8.53	–10.39	–23.53	–2.67	–32.72	–10.88	–8.52	–14.22	–30.48	–16.98
8f	D-827795/1	94.28	95.86	97.66	106.57	99.11	91.34	99.49	93.46	95.63	97.11
8g	D-827792/1	100.76	99.63	104.01	107.01	102.18	96.63	106.70	101.34	97.70	101.56
8h	D-827794/1	96.28	96.26	108.20	100.74	100.82	98.72	107.10	112.77	97.81	101.18
8i	D-827793/1	96.47	96.97	104.55	107.07	102.93	98.06	106.49	109.23	94.68	101.09

Note: *BC: Breast Cancer; PC: Prostate Cancer; RC: Renal Cancer; OC: Ovarian Cancer; CNSC: Central Nervous System Cancer; CC: Colon Cancer; NSCLC: Non-Small Cell Lung Cancer; M: Melanoma; L: Leukemia.

Table 3: Anticancer action of the synthesized compounds against most sensitive cell line

Compound	NSC Code	Range of Growth %	Cell line with the highest sensitivity	Most susceptible cell line's growth percentage	Growth inhibition (GI%)
8a	D-827790/1	85.32 to 119.49	CNSC (SNB-75)	85.32	14.68
			NSCLC (EKVX)	86.00	14.00
			RC (UO-31)	88.87	11.13
			NSCLC (HOP-92)	90.22	9.78
			BC (MCF7)	91.25	8.75
8b	D-827787/1	76.33 to 128.42	NSCLC (EKVX)	76.33	23.67
			RC (UO-31)	83.25	16.75
			CNSC (SNB-75)	84.12	15.88
			NSCLC (HOP-92)	85.54	14.46
			BC (MCF7)	89.82	10.18
8c	D-827788/1	74.65 to 127.22	NSCLC (EKVX)	74.65	25.35
			BC (MCF7)	85.98	14.02
			CNSC (SNB-75)	90.21	9.79
			RC (UO-31)	90.30	9.70
			NSCLC (NCI-H522)	91.13	8.87

(Continued)

Table 3 (continued)

Compound	NSC Code	Range of Growth %	Cell line with the highest sensitivity	Most susceptible cell line's growth percentage	Growth inhibition (GI%)
8d	D-827789/1	85.70 to 133.34	CC (HCT-116)	85.70	14.30
			NSCLC (NCI-H23)	89.40	10.60
			NSCLC (EKVX)	89.48	10.52
			RC (CAKI-1)	90.00	10.00
			NSCLC (NCI-H522)	91.36	8.64
8e	D-827791/1	–86.48 to 95.02	L (SR)	0.31	99.69
			NSCLC (EKVX)	0.60	99.40
			OC (OVCAR-5)	1.06	98.94
			M (SK-MEL-28)	2.62	97.38
			CC (SW-620)	3.64	96.36
			CNSC (SNB-19)	9.50	90.50
			BC (MDA-MB-231/ ATCC)	10.25	89.75
			CNSC (U251)	–13.15	Cytotoxic
			RC (UO-31)	–12.71	Cytotoxic
			M (UACC-257)	–11.92	Cytotoxic
			CNSC (SF-295)	–11.72	Cytotoxic
			BC (MCF7)	–9.04	Cytotoxic
			CC (OVCAR-8)	–8.65	Cytotoxic
			(KM12)	–7.12	Cytotoxic
			RC (RXF 393)	–86.48	Cytotoxic
8f	D-827795/1	70.82 to 112.29	RC (UO-31)	74.18	25.82
			NSCLC (EKVX)	76.89	23.11
			CNSC (SNB-75)	80.05	19.95
			NSCLC (HOP-92)	82.44	17.56
			CC (HCT-116)	83.26	16.74
8g	D-827792/1	71.26 to 116.47	RC (UO-31)	71.26	28.74
			RC (CAKI-1)	81.62	18.38
			NSCLC (HOP-92)	86.26	13.74
			NSCLC (HOP-62)	91.50	8.50
			BC (MCF7)	92.12	7.88
8h	D-827794/1	70.82 to 112.29	RC (UO-31)	77.94	22.06
			CNSC (SNB-75)	82.04	17.96
			BC (BT-549)	85.04	14.96
			NSCLC (HOP-92)	88.44	11.56
			RC (CAKI-1)	88.64	11.36

(Continued)

Table 3 (continued)

Compound	NSC Code	Range of Growth %	Cell line with the highest sensitivity	Most susceptible cell line's growth percentage	Growth inhibition (GI%)
8i	D-827793/1	81.11 to 120.60	RC (UO-31)	81.11	18.89
			NSCLC (EKVX)	81.61	18.39
			CNSC (SNB-75)	83.30	16.70
			BC (BT-549)	84.33	15.67
			NSCLC (HOP-92)	86.02	13.98

Note: *60 cell lines assay in 1 dose 10^{-5} M conc.

Table 4: Five-dose assay of compound 8e against 60 human cancer cell lines

Panel	Cell line	8e		
		GI_{50}	TGI	LC_{50}
BC	HS 578T	2.05	5.97	>100
	MDA-MB-231/ATCC	2.12	4.77	20.4
	MCF7	1.44	5.21	44.9
	MDA-MB-468	1.85	4.53	34.00
	T-47D	2.31	6.70	86.8
	BT-549	1.44	4.85	43.8
PC	PC-3	0.794	3.63	>100
	DU-145	2.21	5.91	>100
RC	CAKI-1	1.27	3.65	11.4
	ACHN	1.50	2.90	5.62
	A498	1.70	3.31	6.46
	786-0	1.97	4.21	9.01
	TK-10	2.10	6.81	>100
	SN12C	1.84	5.40	35.6
	UO-31	1.27	2.62	5.38
	RXF 393	1.21	2.51	5.23
OC	OVCAR-4	2.23	6.36	>100
	OVCAR-3	2.29	5.69	51.4
	IGROV1	0.414	1.62	6.85
	SK-OV-3	1.20	3.46	–
	NCI/ADR-RES	1.77	5.39	>100
	OVCAR-8	2.80	–	>100
	OVCAR-5	1.42	4.19	16.7

(Continued)

Table 4 (continued)

Panel	Cell line	8e		
		GI_{50}	TGI	LC_{50}
CNSC	SF-268	2.11	5.16	>100
	SF-295	2.17	5.11	16.1
	SF-539	1.82	4.48	13.1
	SNB-19	2.31	6.50	60.3
	SNB-75	2.03	5.61	>100
	U251	2.36	9.14	43.8
NSCLC	HOP-62	2.50	6.58	>100
	EKVX	2.10	5.52	27.6
	A549/ATCC	2.57	8.02	>100
	NCI-H522	1.28	4.12	36.9
	NCI-H460	1.21	3.26	8.78
	NCI-H322M	2.16	5.85	25.9
	NCI-H23	1.14	3.45	>100
	HOP-92	1.68	4.56	17.3
	NCI-H226	1.64	4.46	>100
CC	HCT-116	1.71	5.02	50.9
	HCC-2998	1.98	3.91	7.73
	COLO 205	1.65	4.60	30.6
	SW-620	0.491	7.69	75.9
	HT29	0.797	3.26	39.6
	HCT-15	0.845	5.35	58.5
	KM12	2.61	7.99	62.5
M	MDA-MB-435	1.68	3.10	5.71
	M14	1.50	3.28	7.14
	MALME-3M	1.51	3.06	6.16
	LOX IMVI	0.411	1.67	4.82
	UACC-62	0.574	2.14	6.10
	UACC-257	2.50	7.47	>100
	SK-MEL-5	1.68	3.17	6.00
	SK-MEL-28	1.78	3.71	7.74
	SK-MEL-2	2.01	4.84	27.8

(Continued)

Table 4 (continued)

Panel	Cell line	8e		
		GI_{50}	TGI	LC_{50}
L	SR	0.646	>100	>100
	RPMI-8226	2.51	6.79	>100
	MOLT-4	0.570	>100	>100
	K-562	1.89	>100	>100
	HL-60(TB)	2.57	14.2	>100
	CCRF-CEM	0.585	>100	>100

Note: *Values are in μM.

Table 5: Log GI_{50} values of 8e

Compound	CNSC	CC	NSCLC	L	RC	M	PC	BC	OC	MG_MID*
8e	–5.67	–5.90	–5.76	–5.92	–5.80	–5.87	–5.88	–5.73	–5.82	–5.82
Melphalan [50]	–5.12	–5.11	–5.17	–5.48	–4.99	–5.08	–4.49	–4.79	–5.18	–5.09

Note: *Full panel mean graph midpoint.

3.2.2 Antioxidant Activity

In vitro antioxidant activity of the synthesized derivatives was investigated in terms of percentage (%) inhibition by DPPH assay using ascorbic acid as a reference (Fig. 2). By plotting concentrations against percent inhibition of the test compound, the IC_{50} value of synthesized compounds was calculated. Only a few synthesized compounds had significant antioxidant activity, while others had moderate to strong antioxidant activity, according to the results. The antioxidant properties of the compounds **8e** and **8g** were excellent, with IC_{50} values and inhibition percentages equivalent to ascorbic acid (Fig. 3). The findings of the antioxidant screening are presented in Table 6.

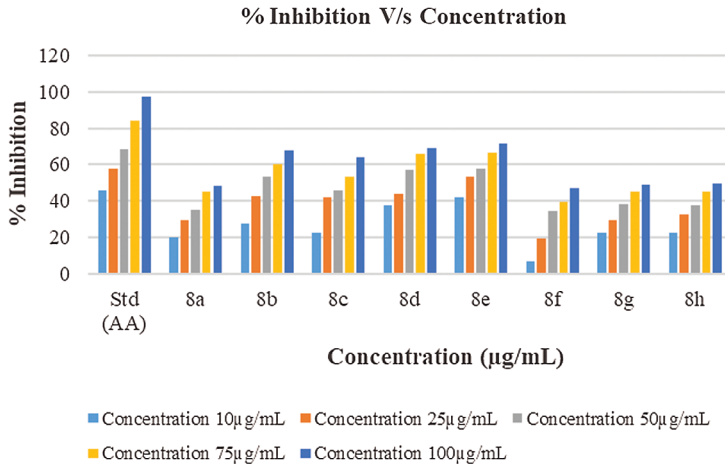


Figure 2: Antioxidant behavior of synthesized derivatives and Ascorbic acid

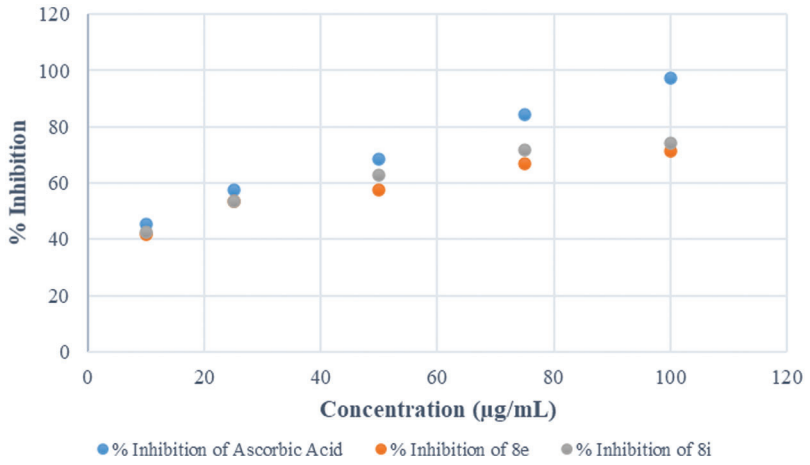


Figure 3: Antioxidant behavior of potent derivatives and Ascorbic acid

Table 6: Percent inhibition and IC_{50} values of synthesized derivatives

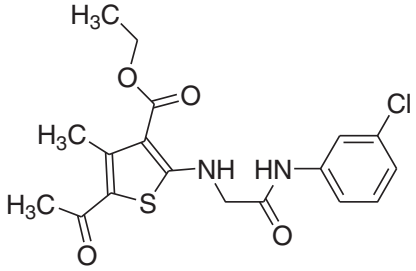
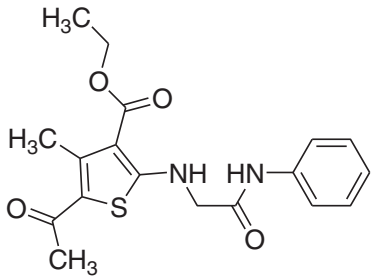
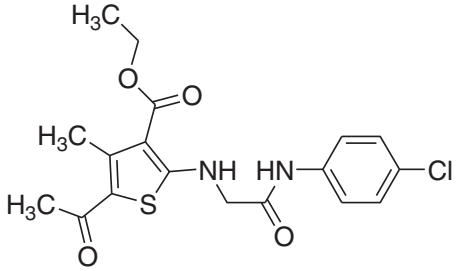
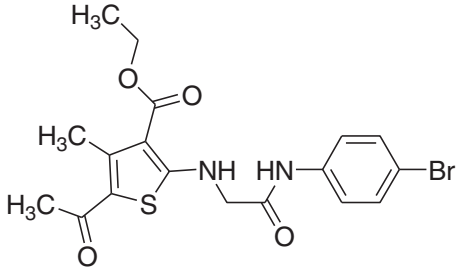
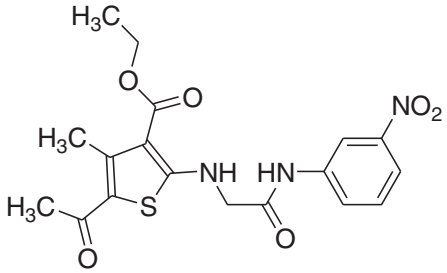
Compounds	Concentration*					IC_{50}
	10	25	50	75	100	
8a	19.85	29.32	34.77	45.09	47.97	99.25 ± 0.303
8b	27.24	42.4	53.09	60.18	67.87	51.54 ± 0.276
8c	22.71	41.69	45.67	53.22	64.04	63.38 ± 0.186
8d	37.26	43.71	57.34	65.6	69.17	39.38 ± 0.257
8e	41.83	53.32	57.48	66.78	71.43	25.49 ± 0.297
8f	6.76	19.45	34.27	39.55	47.21	99.29 ± 0.308
8g	22.16	29.32	38.34	44.78	49.12	96.55 ± 0.301
8h	22.47	32.42	37.62	45.16	49.79	95.26 ± 0.228
8i	42.58	53.52	62.79	71.7	74.15	20.74 ± 0.282
Standard**	45.49	57.70	68.58	84.17	97.17	15.31 ± 0.216

Note: *µg/mL, **Ascorbic acid; Values are expressed as mean ± standard deviation.

3.3 In Silico Studies

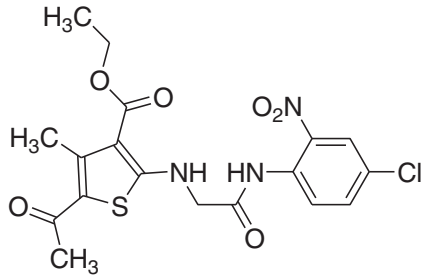
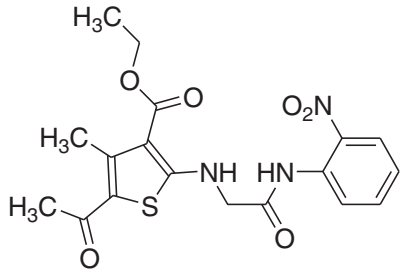
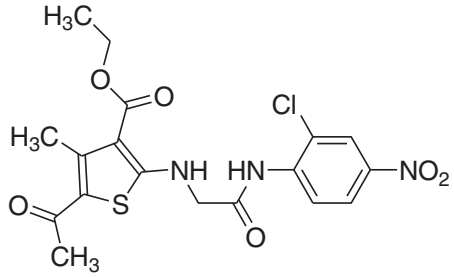
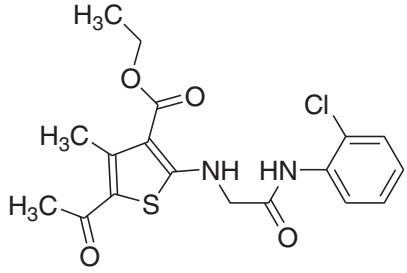
To explore prospective RORγt receptor inhibitor molecules, a molecular docking simulation-based *in silico* virtual screening was carried out. A ligand library of nine thiophene derivatives was virtually screened against the validated RORγt receptor. The binding affinity of all ligand molecules was determined by calculating the binding energy for each ligand's top-ranked pose, and the interactions of docked compounds were visualized. Against the RORγt receptor, the outcomes of AutoDock-based molecular docking simulations of the nine ligand molecules are summarized in Table 7. Based on the lowest binding energy, the best ligand molecule was chosen. As per empirical information, the free binding energy should be between –5 to –15 kcal/mol. Figs. 4 and 5 show a ligand interaction diagram (2D) and a pictorial representation (3D) of 8e.

Table 7: Outcomes of AutoDock-based molecular docking simulations against the RORγt receptor

S. No.	Compound	Chemical structure	Binding energy*
1	8a		–8.05
2	8b		–7.73
3	8c		–8.16
4	8d		–7.10
5	8e		–8.37

(Continued)

Table 7 (continued)

S. No.	Compound	Chemical structure	Binding energy*
6	8f		–7.89
7	8g		–8.64
8	8h		–8.83
9	8i		–8.75

Note: **Binding energy in kcal/Mol.

3.4 Structure-Activity Relationship (SAR) Studies

Based on antioxidant and anti-cancer data, the SAR of synthesized thiophene derivatives may be interpreted as follows:

- The results of the antiproliferative research indicated that electron-withdrawing groups at the *meta*-position are required for activity, which complemented the *in-silico* docking analysis precisely.
- In compounds containing an electron-withdrawing group in the *ortho*- and *meta*-positions of the phenyl group, significant antioxidant activity was observed.

- The antioxidant function of the synthesized compounds may work synergistically to combat the enhanced oxidative stress in cancerous conditions.
- As a result of the resonating effect, the derivative having a nitro group at *meta*-position in the aromatic ring exhibited substantial anticancer and antioxidant activities.

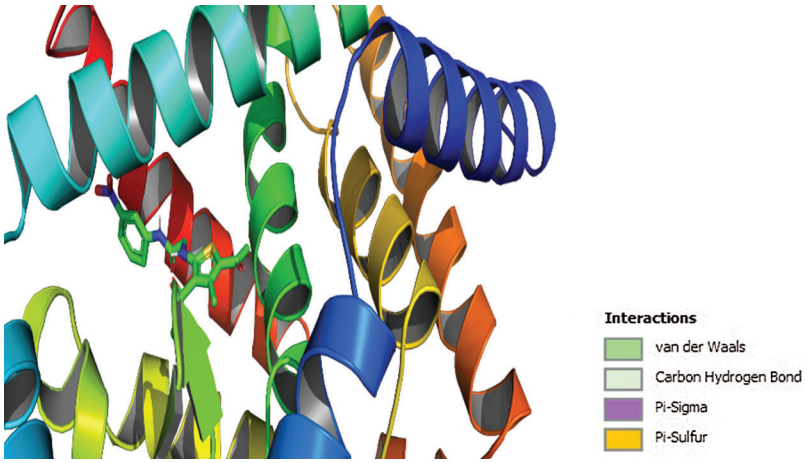


Figure 4: Ligand interaction diagram (2D) of 8e

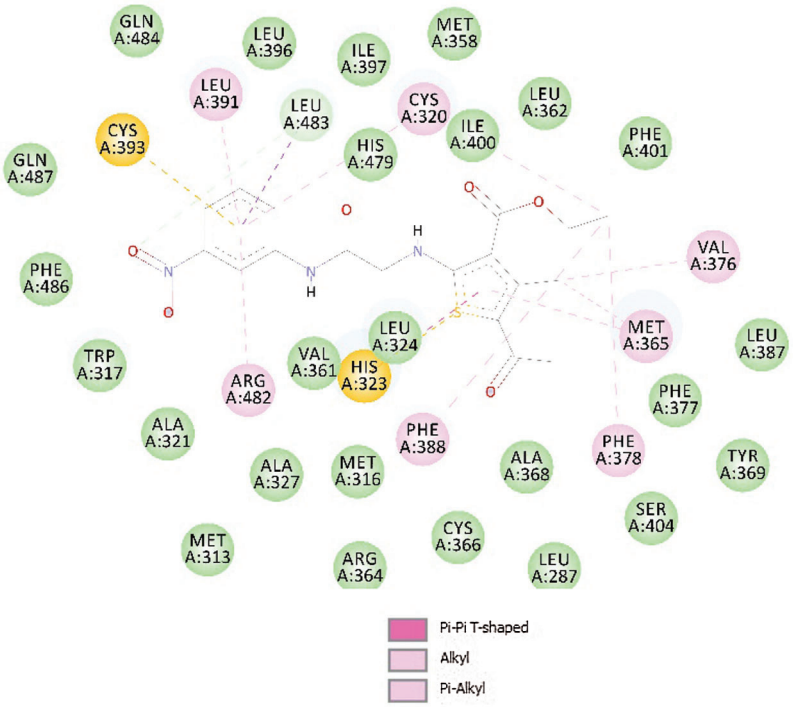


Figure 5: Pictorial presentation (3D) of 8e

4 Conclusion

To summarize, this paper discusses the synthesis, pharmacological prospects, and *in silico* studies of novel thiophene derivatives. *In vitro* antiproliferative and antioxidant functions of synthesized compounds were assessed. Among the synthesized derivatives, compound 8e revealed notable anticancer activity, while compounds 8d, 8e, and 8i evinced promising antioxidant activity. In addition, inside the binding pocket, all the compounds revealed a good docking score in the molecular docking study. The findings of the *in-silico* study correspond to the cytotoxicity tests. In compound 8e, the presence of an electron-withdrawing (*m*-NO_2) group at benzylidene ring conferred upon it the highest anticancer and antioxidant activity. It is suggested that antioxidant activity may play a role in cancer progression control, while cytotoxic ability may be utilized over cancer cells, leading them to apoptosis and cell death. These remarkable biological screening results of synthesized derivatives will help provide a strong basis in this field and pave the way for the establishment of effective therapeutics.

Ethics Approval and Informed Consent Statement: Not Applicable.

Availability of Data and Materials: The data supporting the article is available within the article.

Authors' Contribution: All authors contributed equally to this work. Raghav Mishra, Nitin Kumar and Neetu Sachan prepared the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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