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Synthesis, Cytotoxicity Assessment and Antioxidant Activity of Some New 4-Thiazolidinone Derivatives Containing 2-Methyl Furane Ring

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ABSTRACT: 4-Thiazolidinone derivatives bearing a 2-methyl furan ring are an important class of heterocyclic compounds with wide structural variability. These heterocyclic compounds are of increasing interest in medicinal chemistry due to their reported wide spectrum of pharmacological activities, including antioxidant and anticancer effects. In this study, a series of new 4-thiazolidinone derivative compounds bearing a furan ring system were synthesized, and their antioxidant and cytotoxic activities were investigated. Thiazolidinone compounds (**2a-e**, **3a-j**) were synthesized by refluxing hydrazines and appropriate aldehydes or ketones with mercaptoacetic acid or 2-sulfanylpropanoic acid in toluene using Dean-Stark apparatus for 4 hours. The new compounds (**2a-e**) were characterized by IR, ^{1}H NMR and ^{13}C NMR (APT) spectral studies. The cytotoxic activities of all the compounds (**2a-e**, **3a-j**) against lung (A549), prostate (PC-3) and endometrial (ECC-1) cancer cells, and non-cancerous Human Umbilical Vein Endothelial Cells (HUVEC), were tested in vitro using 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium inner salt (MTS) assay. The antioxidant potentials of the compounds were evaluated by the Ferric Reducing Antioxidant Power (FRAP) and the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assays. The findings revealed that **3g** demonstrated the highest DPPH· scavenging activity and ferric reducing antioxidant power. Furthermore, **3h** exhibited the highest cytotoxic activity, however, **3d** demonstrated selective cytotoxicity toward ECC-1 cells. In conclusion, the compounds displayed weak antioxidant properties but comparatively stronger cytotoxic effects. The selectivity of **3d** suggests potential for further investigations.

KEYWORDS: 4-thiazolidinone; furan; synthesis; cytotoxic activity; antioxidant activity.

1. INTRODUCTION

Free radicals are reactive, high-energy, unstable atomic or molecular structures that contain an unpaired electron pair in their outer orbitals [1]. The free radicals in the organism can occur through endogenous sources such as metabolism, respiration, and enzyme reactions, as well as through exogenous sources such as air pollution, ultraviolet rays, cigarette smoke, and drugs (mycotoxins, pesticides) [1, 2]. Reactive oxygen species (ROS) are the most common free radicals generated in living organisms [3]. High density of ROS can damage the various biomolecules including DNA, protein and lipids, resulting in oxidative stress. Cumulative oxidative stress can cause weakening and loss of immunity in various pathophysiological conditions, including cancer. Antioxidant compounds are substances that stop or slow down the oxidation reactions caused by these free radical structures. They show their effects by neutralizing free radical structures and preventing the body from being affected by them. Research has shown that substances with antioxidant activity play an important role in preventing many diseases thought to be caused by oxidative stress [4-6]. Although ROS levels promote oncogenic signaling, excessive accumulation can surpass cellular tolerance and trigger apoptosis or necrosis. This paradox has increased interest in molecules that reduce oxidative stress or selectively increase ROS beyond lethal limits to induce cell death [7]. Antioxidant molecules protect cells from the carcinogenesis process by reducing the accumulation of free radicals and oxidative damage [8,9]. On the contrary, cytotoxic molecules cause cell death in cells through various mechanisms. Cytotoxic compounds do not show selective properties as they cause cell death in both cancerous and non-cancerous cells [10]. The lack

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of this selectivity leads to undesirable side effects [10]. Thus, the compounds have antioxidant properties and cytotoxic effects emerge as valuable candidates for therapeutic development [11].

Furan ring is a five-membered heterocyclic ring consisting of four carbon atoms and one oxygen atom [12]. Today, there are many drug molecules containing furan ring system in therapies with antimicrobial, antiprotozoal, antifungal, antidepressant activity, anti-Parkinson, antihypertensive, antiarrhythmic, antiulcer, anticancer, analgesic and anti-inflammatory effects [13]. Due to the optical and excellent charge-carrying properties of the furan ring system has antimicrobial (antibacterial, antifungal and antiviral) and central nervous system (antidepressant, anxiolytic, anticonvulsant, antiparkinson and Alzheimer's disease effects) activities in the treatment. Compounds carrying furan ring system play an important role in the activity of many derivatives with muscle relaxant, antiglaucoma, cardiovascular, antiulcer, anti-inflammatory, analgesic, antioxidant and anticancer properties [14-17].

Otherwise, the thiazolidinone ring system is a five-membered heterocyclic ring system containing a nitrogen and a sulfur atom, and especially the thiazolidin-4-one ring system has significant potential to develop new molecules or improve the pharmaceutical profiles of existing candidate molecules. There are many compounds with thiazolidinone ring system that have antidiabetic [18], antioxidant [19-21], antituberculosis [22], antimicrobial [23,24], anticonvulsant [25], anticancer [26,27], antiviral [28] and anti-inflammatory [29] properties. Many antidiabetic compounds currently used in treatment carry the thiazolidine-2,4-dione structure (Pioglitazone, Rosiglitazone, etc.), and have a high affinity for peroxisome proliferator-activated receptor gamma (PPARγ) [30].

In light of these findings, a series of novel hybrid compounds incorporating both furan and thiazolidine ring systems were designed and synthesized. Their structures were fully characterized, and the synthesized furan derivatives were evaluated for their antioxidant and cytotoxic activities. Additionally, the influence of the 4-thiazolidinone and spirothiazolidinone ring systems on the biological activities was assessed.

2. RESULTS AND DISCUSSION

2.1. Chemistry

The synthetic pathway for the preparation of novel 2-methylfuran-based 4-thiazolidinones (**2a–e**) is illustrated in Figure 1. In this study, new 4-thiazolidinone derivatives incorporating a 2-methylfuran ring were synthesized via a one-pot reaction. The refluxing of hydrazines and an appropriate aldehyde with mercaptoacetic acid acid or 2-sulfanylpropanoic acid in toluene using a Dean–Stark apparatus for 4 h, gave the desired 4-thiazolidinones. The compounds **3a-j** have been previously described by Apaydın et al. (Figure 2) [28]. The novel compounds (**2a-e**) were characterized by elemental analysis and IR, ^{1}H NMR and ^{13}C NMR (APT) spectral studies.

The IR spectra of **2a-e** showed characteristic amide absorption bands at 3284-3188 cm^{-1} (NH), 1726-1693 cm^{-1} (C=O), 1678-1643 cm^{-1} (NH-C=O), which confirmed the formation of 4-thiazolidinones.

The ^{1}H NMR spectra of the synthesized compounds (**2a-d**) showed characteristic broad singlets of the benzamide NH group at δ 10.30-10.26 ppm. The 4-thiazolidinone C2-H of **2a-f** was observed at about δ 5.91-5.88 ppm as a doublet or a broad singlet. For the 4-thiazolidinone derivatives (**2a–c**) synthesized by the reaction of the hydrazide with various aldehydes and thioglycolic acid, the appearance of the C5 methylene (CH_2) proton signals as two separate doublets of doublets at δ 3.97–3.79 ppm confirmed the formation of the thiazolidinone ring. In contrast, for the 4-thiazolidinone derivatives (**2d-f**) obtained from the reaction of the hydrazide with various aldehydes and thiolactic acid, the methyl protons attached to the C5 methyl group appeared as a doublet at 1.55-1.53 ppm, providing characteristic evidence for successful ring closure. In the ^{1}H-NMR spectrum, the compound **2f** exhibited double peaks due to diastereomers observed in 4-thiazolidinone derivatives containing two chiral centers [31]. CONH protons were observed as two separate singlets at δ 10.29 and 10.26 ppm. The newly synthesized 4-thiazolidinone derivatives possess chiral centers at C-2 and C-5, and they were most likely obtained as a mixture of cis and trans diastereomers. Most of the proton resonances appeared as duplicate signals.

APT spectra of **2c** showed two downfield signals at about δ 169.7 and 162.0 ppm due to the carbonyl carbon absorptions.

2.2. Antioxidant activities of the compounds

The antioxidant potentials of the compounds were evaluated using both the FRAP and DPPH scavenging activity assays. Based on the DPPH radical scavenging assay, **3g** reached 50% inhibition within the tested concentration range, with an EC_{50} value of 863.10 ± 24.53 μM. In contrast, **3i** did not reach 50% inhibition

even at the highest tested concentration (1000 µM), exhibiting only 31.00 ± 0.54% inhibition, while the remaining compounds showed no detectable DPPH radical scavenging activity. In the FRAP assay, in which higher values indicate greater ferric-reducing capacity, compounds **2a**, **2c**, **2d**, and **3g–3j** exhibited measurable activity. **3g** exhibited the highest FRAP value among the tested compounds (0.63 ± 0.03 mM Fe^{2+}). Overall, **3g** showed the strongest antioxidant activity among the tested compounds in both assays; however, its activity was considerably lower than that of the reference compound quercetin (Table 1).

Table 1. Antioxidant activities of the compounds.

	DPPH (EC_{50}) (µM)	FRAP (mM Fe^{+2})*
2a	-	0.13 ± 0.03
2b	-	-
2c	-	0.13 ± 0.01
2d	-	0.11 ± 0.01
2e	-	-
2f	-	-
3a	-	-
3b	-	-
3c	-	-
3d	-	-
3e	-	-
3f	-	-
3g	863.10 ± 24.53	0.63 ± 0.03
3h	-	0.15 ± 0.02
3i	>1000	0. 30 ± 0.02
3j	-	0.14 ± 0.01
Quercetin	5.35 ± 0.42	3.75 ± 0.09

The values are expressed as the mean ± standard deviation, based on at least three independent experiments.
* The compounds and quercetin were tested at a concentration of 7.5 mM and 0.66 mM, respectively.
EC_{50}; Half maximal effective concentration.

2.3. Cytotoxic activities of the compounds

In the present study, the cytotoxic effects of the compounds were assessed against the HUVEC, A549, PC-3, and ECC-1 cells, with the results summarized in Table 2. Overall, most compounds exhibited relatively low cytotoxic activity, although generally lower IC_{50} values were observed against ECC-1 cells. Among the tested compounds, 3d, 3h, and 3i exhibited the lowest IC_{50} values. However, comparison with the HUVEC data indicated that their cytotoxic effects were not uniformly selective toward cancer cells. **3h** exhibited the lowest IC_{50} values overall; however, its comparable cytotoxicity toward HUVEC, A549, and PC-3 cells indicated broad, non-selective cytotoxicity rather than preferential activity against cancer cells. **3i** also showed limited selectivity, with a selectivity index of 1.50 against ECC-1 cells. In contrast, **3d** exhibited greater activity against ECC-1 cells and a selectivity index of 2.43. **3e** also showed some preferential activity toward ECC-1 cells, with a selectivity index greater than 2.37, although its cytotoxic potency was lower than that of **3d**. Thus, **3d** demonstrated the most favorable balance between cytotoxic potency and selectivity toward ECC-1 cells among the investigated compounds. Although a clear structure–activity relationship could not be established, compounds bearing the spirothiazolidinone scaffold generally exhibited greater cytotoxicity than the related 4-thiazolidinone analogs. However, their effects on HUVEC cells indicated that this increased cytotoxicity was not consistently cancer-selective.

Table 2. Cytotoxic activities of the compounds on HUVEC, A549, PC-3, and ECC-1 cells.

	IC_{50} (μM)			
	HUVEC	A549	PC-3	ECC-1
2a	>250	>250	>250	>250
2b	>250	>250	223.80 ± 2.36	149.50 ± 6.95
2c	>250	>250	>250	159.97 ± 7.53
2d	>250	>250	>250	>250
2e	204.69 ± 4.70	>250	203.38 ± 3.15	149.75 ± 2.15
2f	>250	>250	>250	>250
3a	>250	>250	>250	>250
3b	>250	>250	>250	178.26 ± 4.34
3c	>250	>250	>250	129.71 ± 4.66
3d	101.78 ± 1.90	95.08 ± 1.24	91.75 ± 1.46	41.94 ± 1.06
3e	>250	>250	>250	105.58 ± 1.46
3f	245.60 ± 4.32	187.44 ± 2.78	228.28 ± 1.93	160.76 ± 4.15
3g	207.62 ± 2.11	225.29 ± 4.91	221.09 ± 3.48	111.79 ± 5.51
3h	44.16 ± 1.47	47.84 ± 0.71	44.38 ± 2.08	34.36 ± 0.46
3i	76.04 ± 0.08	83.10 ± 3.05	93.65 ± 1.73	50.55 ± 2.58
3j	156.07 ± 5.28	190.67 ± 4.01	197.39 ± 5.24	111.07 ± 4.58

The values are expressed as the mean ± standard deviation, based on at least three independent experiments.
IC_{50}: Half maximal inhibitor concentration.

3. CONCLUSION

In this study, a series of sixteen novel 2-methylfuran-containing thiazolidin-4-ones (**2a-e**, **3a-j**) were synthesized by the cyclo-condensation reaction between Schiff bases and mercaptoacetic acid or 2-sulfanylpropanoic acid. The different spectral techniques and the elemental analysis confirmed the structure of the new compounds (**2a-e**). The findings of this study indicated that **3g** demonstrated the highest DPPH· scavenging activity and ferric reducing antioxidant power; however, these activities were still markedly low. The compounds also displayed generally low cytotoxicity, with **3h** exhibiting the highest activity and **3d** demonstrating selective cytotoxicity toward ECC-1 cells. In conclusion, the compounds displayed weak antioxidant properties but comparatively stronger cytotoxic effects.

4. MATERIALS AND METHODS

4.1. Chemicals

Dulbecco's Modified Eagle Medium (DMEM), DMEM-F12, Roswell Park Memorial Institute (RPMI) and penicillin/streptomycin were obtained from Wisent (Montreal, QC, Canada). Fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium inner salt (MTS) and quercetin were sourced from Capricorn (Ebsdorfergrund, Germany), Promega (Madison, USA) and Fluka (Buchs, Switzerland), respectively. 2,4,6-tripyridyl-s-triazine (TPTZ) and penazine methosulfate (PMS) were purchased from Sigma/Merck (St. Louis, USA/Darmstadt, Germany). Mercaptoacetic acid, 2-sulfanylpropanoic acid, and toluene were obtained commercially from Merck (Darmstadt, Almanya). All other chemicals were of analytical grade and purchased from Sigma-Aldrich (Darmstadt, Germany) or Merck (Darmstadt, Germany).

Melting points (M.p.) were determined on a Büchi 530 capillary melting point apparatus in open capillaries and were uncorrected. Infrared (IR) spectra were recorded on a Pelkin Elmer 1600 FT infrared spectrophotometer in potassium bromide pellets (γ in cm^{-1}). ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance Neo 500 MHz (Germany) spectrophotometer. Elementary analyses were performed on a Leco CHNS 932 (St. Joseph, MI, USA) elemental analyzer.

4.2. General synthesis of *N*-(substitue 4-oxo-1,3-thiazolidine-3-yl)-2-methylfuran-3-carboxamide compounds (2a-f)

To a solution of 2-methylfuran-3-carbohydrazide (0.005 mol) in the dry toluene (30 mL) was added an appropriate aldehyde (0.005 mol) and the mixture was refluxed for 4 hours and 1.5 ml mercaptoacetic acid or 2-mercaptopropionic acid was added to the solution and the resulting solution was refluxed for 4-8 h. using a Dean Stark water separator. Excess toluene was evaporated in vacuo. The resulting residue was triturated with saturated $NaHCO_3$ solution. The solid thus obtained was washed with water, dried and recrystallized from ethanol [31].

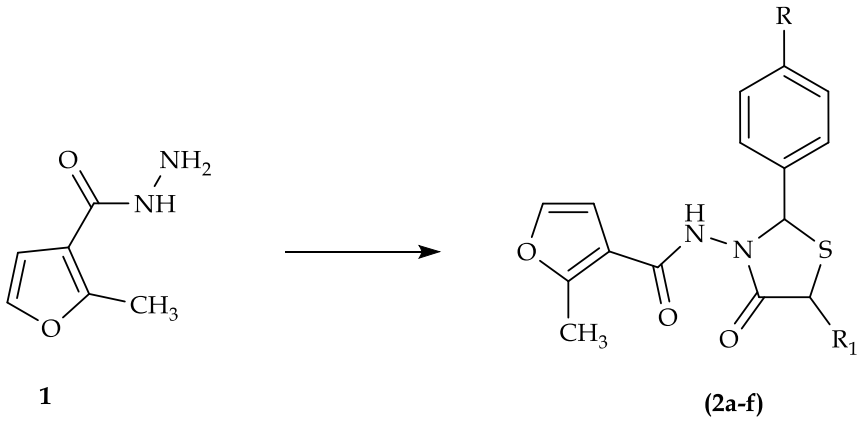


Figure 1. Synthesis of **(2a-f)**. Reagents and conditions: (non)substituted benzaldehyde, mercaptoacetic acid or 2-mercaptopropionic acid, dry toluene, reflux, 4 h.

4.2.1. 3-[(2-methylfuran-3-yl)carbonyl]-2-phenyl-1,3-thiazolidin-4-one (2a)

White powder (%85) mp 175-176 °C; IR (KBr) υ (cm^{-1}): 3188 (NH), 1726 (CO), 1643 (NHCO). ^{1}H-NMR (DMSO-d_6/500MHz): 10.26 (1H, s, NH), 7.52 (d, *J* = 2.1 Hz, fur-H), 7.47-7.33 (5H, m, benz-H), 6.74 (1H, d, *J* = 2.0 Hz, fur-H), 5.90 (1H, d, *J* = 1.5 Hz, tyz-C_2-H), 3.92 (1H, dd, *J* = 16.0, 1.8 Hz, tyz-C_5-H), 3.80 (1H, dd, *J* = 16.0, 0.9 Hz, tyz-C_5-H), 2.45 (3H, s, CH_3). Anal. calcd. for $C_{15}H_{14}N_2O_3S$ (302.34) C: 59.59, H: 4.67, N: 9.27. Found C: 59.42, H: 4.88, N: 9.32.

4.2.2. 2-(4-chlorophenyl)-3-[(2-methylfuran-3-yl)carbonyl]-1,3-thiazolidin-4-one (2b)

White powder (%88) mp 131-134 °C; IR (KBr) υ (cm^{-1}): 3230 (NH), 1701 (CO), 1668 (NHCO). ^{1}H-NMR (DMSO-d_6/500MHz): 10.26 (1H, s, NH), 7.55-7.43 (5H, m, fur-H and benz-H), 6.73 (1H, d, *J* = 2.0 Hz, fur-H), 5.90 (1H, d, *J* = 1.6 Hz, tyz-C_2-H), 3.94 (1H, dd, *J* = 15.9, 1.8 Hz, tyz-C_5-H), 3.79 (1H, dd, *J* = 16.1, 0.8 Hz, tyz-C_5-H), 2.46 (3H, s, CH_3). Anal. calcd. for $C_{15}H_{13}ClN_2O_3S$ (336.79) C: 53.49, H: 3.89, N: 8.32. Found C: 53.33, H: 3.96, N: 8.46.

4.2.3. 2-(4-fluorophenyl)-3-[(2-methylfuran-3-yl)carbonyl]-1,3-thiazolidin-4-one (2c)

White crystal (%80) mp 130-132 °C; IR (KBr) υ (cm^{-1}): 3284 (NH), 1708 (CO), 1678 (NHCO). ^{1}H-NMR (DMSO-d_6/500MHz): 10.24 (1H, s, NH), 7.58-7.50; 7.26-7.18 (5H, m, fur-H and benz-H), 6.73 (1H, d, *J* = 2.1 Hz, fur-H), 5.91 (1H, d, *J* = 1.6 Hz, tyz-C_2-H), 3.93 (1H, dd, *J* = 15.9, 1.8 Hz, tyz-C_5-H), 3.79 (1H, dd, *J* = 15.9, 0.9 Hz, tyz-C_5-H), 2.45 (3H, s, CH_3). ^{13}C-NMR (APT) (DMSO-d_6/125MHz): 169.7 (CO), 162.7 (d, *J*=243.7 Hz, ph-C4), 162.0 (NHCO), 158.0 (fur-C2), 141.6 (fur-C5), 135.0 (d, *J*= 2.5 Hz, ph-C1), 130.5 (d, *J*= 8,7 Hz, ph- C2,6), 115,9 (d, *J*=21.0 Hz, ph- C3,5), 113.3 (fur-C3), 109.2 (fur-C4), 61.6 (tyz-C5), 29.7 (tyz-C2), 13.6 (fur-CH_3). Anal. calcd. for $C_{15}H_{13}FN_2O_3S$ (320.33) C: 56.24, H: 4.09, N: 8.74. Found C: 56.02, H: 4.34, N: 8.46.

4.2.4. 5-methyl-3-[(2-methylfuran-3-yl)carbonyl]-2-phenyl-1,3-thiazolidin-4-one (2d)

White powder (%88) mp 163-166 °C; IR (KBr) υ (cm^{-1}): 3234 (NH), 1693 (CO), 1666 (NHCO). ^{1}H-NMR (DMSO-d_6/500MHz): 10.30 (1H, s, NH), 7.51 (1H, d, *J* = 2.1 Hz, fur-H), 7.49-7.32 (5H, m, benz-H), 6.77-6.70

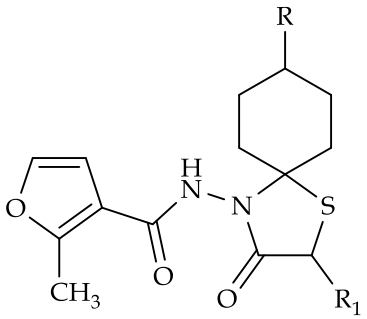
(1H, m, fur-H), 5.88 (1H, d, *J* = 1.0 Hz, tyz-C_2-H), 4.10 (1H, qd, *J* = 6.9, 1.0 Hz, tyz-C_5-H), 2.45 (3H, s, CH_3), 1.55 (3H, d, *J* = 6.7 Hz, tyz-C_5-CH_3). Anal. calcd. for $C_{16}H_{16}N_2O_3S$ (316.37) C: 60.74, H: 5.10, N: 8.85. Found C: 60.52, H:5.23, N: 8.86.

4.2.5. 2-(4-chlorophenyl)-5-methyl-3-[(2-methylfuran-3-yl)carbonyl]-2-phenyl-1,3-thiazolidin-4-one (2e)

White crystal (%81) mp 200-203 °C; IR (KBr) υ (cm^{-1}): 3230 (NH), 1701 (CO), 1668 (NHCO). ^{1}H-NMR (DMSO-d_6/500MHz): 10.30 (1H, s, NH), 7.55-7.42 (5H, m, benz-H and fur-H), 6.73 (1H, dd, *J* = 15.2, 2.1 Hz, fur-H), 5.89 (1H, d, *J* = 1.1 Hz, tyz-C_2-H), 4.11 (1H, qd, *J* = 7.0, 1.0 Hz, tyz-C_5-H), 2.46 (3H, s, CH_3), 1.55 (3H, d, *J* = 7.0 Hz, tyz-C_5-CH_3). Anal. calcd. for $C_{16}H_{15}ClN_2O_3S$ (350.81) C: 54.78, H: 4.31, N: 7.99. Found C: 54.70, H: 4.45, N: 8.14.

4.2.6. 2-(4-fluorophenyl)-5-methyl-3-[(2-methylfuran-3-yl)carbonyl]-2-phenyl-1,3-thiazolidin-4-one (2f)

White powder (%85) mp 192-197 °C; IR (KBr) υ (cm^{-1}): 3234 (NH), 1701 (CO), 1670 (NHCO). ^{1}H-NMR (DMSO-d_6/500MHz): 10.29, 10.26 (1H, 2s, NH), 7.55-7.50; 7.25-7.20 (5H, m, benz-H and fur-H), 6,74, 6.71 (1H, 2d, *J*=2.1 Hz, fur-H), 5.88 (1H, broad s, tyz-C_2-H), 4.18, 4.10 (1H, qd, *J*= 6.9, 1.0 Hz, tyz-C_5-H), 2.46, 2.45 (3H, 2s, CH_3), 1.55, 1.53 (3H, 2d, *J* = 7.0 Hz, tyz-C_5-CH_3). Anal. calcd. for $C_{16}H_{15}FN_2O_3S$ (334.36) C: 57.47, H: 4.52, N: 8.38. Found C: 57.24, H: 4.68, N: 8.45.



(3a-f)

Compound
3a: R= H; R_1= H
3b: R=CH_3; R_1= H
3c: R=C_3H_7; R_1= H
3d: R=$C(CH_3)_3$; R_1= H
3e: R=C_6H_5; R_1= H
3f: R=CH_3; R_1= CH_3
3g: R=C_2H_5; R_1= CH_3
3h: R=C_3H_7; R_1=CH_3
3i: R=$C(CH_3)_3$; R_1=CH_3
3j: R=C_6H_5; R_1=CH_3

Figure 2. Structure of 3a-j (The synthesis of the compounds has been previously described by Apaydın et al. [26])

4.3. General synthesis of *N*-(8-unsubstituted/substıtued-3-oxo-1-thia-4-azaspiro[4.5]decan-4-yl)-2-methylfuran-3-carboxamide compounds (3a-f)

The compounds presented in Figure 2 were synthesized according to the procedure previously described by Apaydın et al. [26].

4.4. Evaluation of antioxidant activity

DPPH radical (DPPH·) scavenging activity

DPPH radical (DPPH·) scavenging potentials of the compounds were assessed according to the method outlined by Brand-Williams et al. (1995) [32]. Quercetin and methanol were used as positive and negative control, respectively. A 10 µL sample was combined with 240 µL DPPH· solution (0.1 mM in methanol) and incubated in the dark at room temperature for 30 min. Following incubation, the reduction in the absorbance was measured at 517 nm. The percentage of DPPH· scavenging activity was then calculated using the formula: DPPH· scavenging activity (%) = [1−(Absorbance of sample / Absorbance of control)] ×100. EC_{50} values were calculated from concentration–response curves generated using the percentage DPPH radical-scavenging activity obtained at different compound concentrations. For compounds that did not reach 50% inhibition at the highest tested concentration of 1000 µM, the EC_{50} value was reported as >1000 µM.

Ferric-reducing antioxidant power (FRAP)

The antioxidant activities of the compounds were also established using the Ferric Reducing Antioxidant Power (FRAP) assay [33], with quercetin used as a positive control. The FRAP working solution

was prepared by combining 0.3 M acetate buffer, 20 mM ferric chloride solution, and 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution (v:v:v, 10:1:1). A 10 µL sample was mixed with 20 µL of appropriate solvent (DMSO for the compounds/ethanol for quercetin) and the mixture was incubated for 10 min at 37°C. Subsequently, 270 µL of FRAP working solution was added and the mixture incubated for an additional 4 min. The absorbance was measured at 593 nm, and the antioxidant powers of the compounds were expressed as mM Fe^{+2} equivalents, calculated from the regression equation derived from $FeSO_4.7H_2O$ standards (0.2–1.5 mM).

4.5. Evaluation of cytotoxic activity

Cell culture

Human cell lines representing lung cancer (A549, CCL-185), prostate cancer (PC-3, CRL-1435), endometrial cancer (ECC-1, CRL-2923), and non-cancerous umbilical vein/vascular endothelium (HUVEC, CRL-1730) were sourced from the American Type Culture Collection. The HUVEC and A549 cell lines were maintained in DMEM, whereas ECC-1 and PC-3 cell lines were cultured in RPMI 1640, and DMEM-12, respectively. All media were supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

MTS cell viability assay

A cell viability assay was conducted using MTS reagent to evaluate the cytotoxic effects of the compounds [34]. HUVEC (1×10^4), A549 (2×10^4), ECC-1 (1×10^4), and PC-3 (2×10^4) cells were seeded in 96-well plates. The following day, the compounds were dissolved in DMSO, and cells were treated with various concentrations of the compounds (300– 2.34 µM) for 72 h. After 72 hours, an MTS/PMS mixture (v:v, 20:1) was added to each well and cells were incubated for 1–4 h at 37 °C. The absorbance was measured using a microplate reader (Biotek, Winooski, VT, USA) at 490 nm. The percentage of cell proliferation inhibition was calculated using the following formula:

$$\text{Inhibition } (\%) = [1 - (\text{Absorbance of test} / \text{Absorbance of control})] \times 100.$$

The IC_{50} values, representing the concentration required for 50% inhibition of cell proliferation, were determined by identifying the x-axis values corresponding to half of the maximum absorbance.

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