

Design, synthesis and biological evaluation of new bis(thiosemicarbazone) derivatives as potential targeted anticancer agents for non-small cell lung cancer

Belgin SEVER^{1*} , Gülşen AKALIN ÇİFTÇİ² , Ahmet ÖZDEMİR¹ ,
Mehlika Dilek ALTINTOP¹ 

¹ Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Anadolu University, 26470 Eskişehir, Turkey.

² Department of Biochemistry, Faculty of Pharmacy, Anadolu University, 26470 Eskişehir, Turkey.

* Corresponding Author. E-mail: belginsever@anadolu.edu.tr (B.S.); Tel. +90-222-335 05 80.

Received: 03 July 2020 / Revised: 17 August 2020/ Accepted: 27 August 2020

ABSTRACT: Thiosemicarbazones represent an important class of ligands for targeted therapy of many types of cancer including non-small cell lung cancer. In order to identify potential antitumor agents for targeted therapy of lung cancer, new bis(thiosemicarbazone) derivatives (**1-11**) were prepared *via* the reaction of 1,4-phenylenebis(thiosemicarbazide) with 5-arylfurfurals. The cytotoxic effects of compounds **1-11** on A549 human lung adenocarcinoma and L929 mouse fibroblast cells were investigated using MTT test. Compounds **1**, **10** and **11** were the most potent anticancer agents in this series on A549 cell line with IC₅₀ values of 14.33±0.47 µg/mL, 11.67±2.49 µg/mL and 16.67±5.56 µg/mL, respectively compared to cisplatin (IC₅₀= 18.33±0.94 µg/mL). Based on their IC₅₀ values for L929 cell line, their anticancer activities were found to be selective. Moreover, flow cytometry-based analyses were performed to examine their effects on apoptosis and mitochondrial membrane potential. The treatment of A549 cells with compounds **1**, **10** and **11** at IC₅₀ concentrations led to the induction of apoptosis along with mitochondrial membrane depolarization. In order to explore their mode of action, compounds **1**, **10** and **11** were evaluated for their inhibitory effects on COX-1 and COX-2 in A549 cells. In particular, *N,N'*-(1,4-phenylene)bis(2-((5-(2,5-dichlorophenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (**10**) was identified as a selective COX-2 inhibitor (6.96% for COX-1 and 54.81% for COX-2). According to these results, compound **10** warrants further *in vitro* and *in vivo* studies as a potential targeted anticancer agent for the management of non-small cell lung cancer.

KEYWORDS: Apoptosis; bis(thiosemicarbazone); cyclooxygenase-2; mitochondrial membrane potential; non-small cell lung cancer.

1. INTRODUCTION

Lung cancer (LC) accounts for the majority of cancer-related mortality with nearly 1.5 million deaths annually, and represents almost 1/5 of all cancer deaths [1]. During the early stages, LC is commonly asymptomatic and therefore nearly 75% of LC cases are diagnosed at advanced or metastatic stages (stage III/IV) leading to a less than 15% 5-year survival rate [2]. Based on pathologic features, LC is split into two main categories, namely non-small cell LC (NSCLC) and small-cell LC (SCLC). NSCLC, the most diagnosed LC type, is accountable for almost 85% of all cases. The late diagnosis of NSCLC causes patients to miss the most opportune time for surgery and thereby radiotherapy and platinum-based chemotherapy become important therapeutic approaches for unresectable NSCLC [3]. Due to the severe toxicity and low specificity of platinum-based conventional chemotherapeutic drugs, targeted therapies exerting selective antitumor action through specific molecular targets with better therapeutic efficacy and lower toxicity have emerged as promising therapeutic options for NSCLC [1-4].

Overexpression of cyclooxygenase-2 (COX-2) participates in the pathogenesis of many types of cancer including NSCLC [5]. COX-2 is released by cancer-associated fibroblasts, macrophage type 2 cells, and cancer cells to the tumor microenvironment. COX-2 induces cancer stem cell-like activity, and promotes apoptotic resistance, inflammation, proliferation, angiogenesis, invasion, and metastasis of tumor cells and it is also associated with cancer cell resistance to chemo and radiotherapy. As a consequence, COX-2-targeted therapy is a promising approach for the management of cancer [5-10].

How to cite this article: Sever B, Akalın Çiftçi G, Özdemir A, Altintop MD. Design, synthesis and biological evaluation of new bis(thiosemicarbazone) derivatives as potential targeted anticancer agents for non-small cell lung cancer. J Res Pharm. 2020; 24(5): 670-680.

Thiosemicarbazones (TSCs) have come into prominence as eligible and worthy candidates for cancer therapy [11,12]. The strong metal-chelating/metal-interacting properties of TSCs have led to the development of potent anticancer drugs due to the fact that tumor cells require essential metals for growth and proliferation. TSCs have an impressive ability to suppress the growth and proliferation of a variety of tumors through the promotion of apoptosis, cell cycle arrest and inhibition of molecular targets involved in the pathogenesis of cancer [11-22]. Over the past decade, TSCs have also been reported to show pronounced inhibitory effects on COX enzymes [23,24].

Taking into account the knowledge obtained so far [18-20] and the potential of TSCs for the treatment of cancer [11-20], herein we reported the preparation of new bis(thiosemicarbazone) derivatives and *in vitro* studies related to their cytotoxic effects on A549 human lung adenocarcinoma and L929 mouse fibroblast (normal) cells. In order to provide a mechanistic insight into their mode of action, the potent and selective anticancer agents in this series were investigated for their effects on apoptosis, mitochondrial membrane potential and COXs.

2. RESULTS AND DISCUSSION

New bis(thiosemicarbazone) derivatives (**1-11**) were obtained *via* a facile and efficient two-step synthetic procedure as depicted in Figure 1. In the first step, 1,4-phenylenebis(thiosemicarbazide) was synthesized *via* the reaction of *p*-phenylene diisothiocyanate with hydrazine hydrate. Finally, the reaction of 1,4-phenylenebis(thiosemicarbazide) with 5-arylfurfurals afforded a new series of bis(thiosemicarbazones) (**1-11**). The Infrared (IR), ¹H Nuclear Magnetic Resonance (NMR), ¹³C NMR, and mass spectral data were used to confirm the structures of compounds **1-11**. In their IR spectra, the N-H stretching vibrations belonging to the thiosemicarbazone group gave rise to the bands in the region 3334.92-3275.13 cm⁻¹. Aromatic and aliphatic C-H stretching bands appeared at 3140.11-3010.88 cm⁻¹ and 2980.02-2953.02 cm⁻¹, respectively. N-H bending, C=N and C=C stretching bands were observed between 1620.21 cm⁻¹ and 1452.40 cm⁻¹. In the ¹H NMR spectra of compounds **1-11**, the characteristic azomethine (-CH=N-) protons were observed as singlets at 8.05-8.18 ppm. Besides, the characteristic NH protons appeared at 9.84-10.06 ppm and 11.92-12.07 ppm, respectively. The protons of furan rings gave rise to the peaks at 7.10-7.41 ppm. In the ¹³C NMR spectra of compounds **1-11**, the azomethine (-CH=N-) and the thiocarbonyl carbons of final compounds were detected at 135.76-136.63 ppm and 175.34-176.26 ppm, respectively. The C₂, C₃, C₄ and C₅ carbons of the furan rings appeared at 148.13-150.98 ppm, 109.07-114.47 ppm, 114.83-115.73 ppm and 149.89-153.57 ppm, respectively [25]. All the protons and carbons of compounds **1-11** were consistent with their proposed structures. Finally, the mass spectral data and elemental analyses were coherent with their molecular formulas.

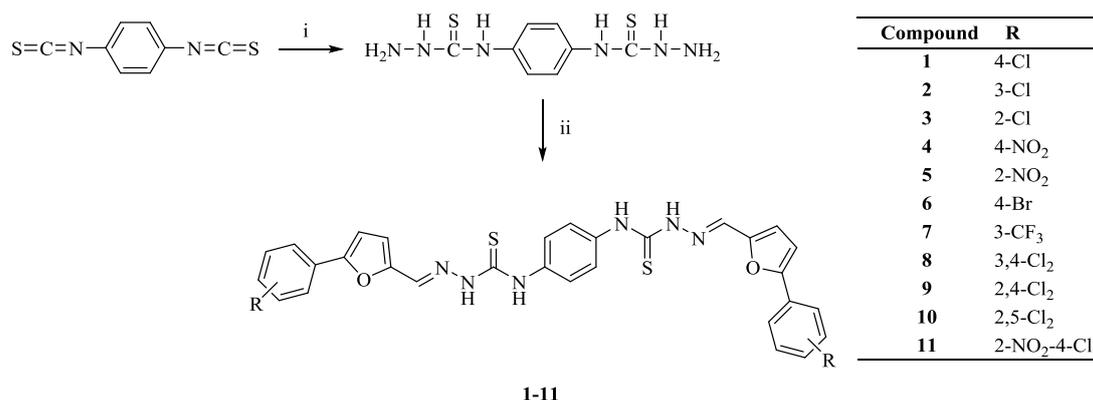


Figure 1. The synthetic route for the preparation of the bis(thiosemicarbazone) derivatives (**1-11**). Reagents and conditions: (i) NH₂NH₂·H₂O, ethanol, rt, 4h; (ii) 5-arylfurfural, ethanol, reflux, 8h.

In order to determine the anticancer effects of compounds **1-11** on A549 human lung adenocarcinoma cell line, MTT assay was performed. Compounds **1**, **10** and **11** were found to be more potent on A549 cell line than cisplatin (IC₅₀= 18.33±0.94 µg/mL). Compounds **1**, **10** and **11** showed significant cytotoxic activity against A549 cell line with IC₅₀ values of 14.33±0.47 µg/mL, 11.67±2.49 µg/mL and 16.67±5.56 µg/mL, respectively. On the other hand, compound **2** exhibited moderate cytotoxic activity with an IC₅₀ value of 68.33±11.79 µg/mL. The chloro substituent was found to be essential for anticancer activity towards A549 cell line. Surprisingly, compounds **3**, **8** and **9** showed no cytotoxic activity against A549 cell line at the tested

concentrations. This outcome pointed out the importance of the position of chloro substituent for anticancer activity. 4-Chloro, 2,5-dichloro and 4-chloro-2-nitro substitutions led to the enhancement of anticancer activity. The effects of compounds **1-11** on L929 mouse fibroblast cell line were investigated using MTT test to assess whether the compounds were toxic or nontoxic to normal cells. The selectivity index (SI) values of compounds **1, 2, 10** and **11** were also determined to compare their selectivity (Table 1). The IC₅₀ values of these agents for L929 cell line were higher than their IC₅₀ values for A549 cell line indicating that their anticancer effects were selective. In particular, the selectivity of compound **11** (SI>30) was found to be noteworthy.

Table 1. IC₅₀ values of the compounds for A549 and L929 cells after 24 h.

Compound	IC ₅₀ (µg/mL)		SI*
	A549 Cell line	L929 Cell line	
1	14.33±0.47	231.67±42.52	16.17
2	68.33±11.79	139.00±15.56	2.03
3	>500	340.00±17.32	ND
4	>500	103.00±18.38	ND
5	>500	24.50±7.78	ND
6	>500	31.67±7.64	ND
7	>500	336.67±37.86	ND
8	>500	7.50±0.87	ND
9	>500	101.67±20.21	ND
10	11.67±2.49	30.67±5.13	2.63
11	16.67±5.56	>500	>30.00
Cisplatin	18.33±0.94	NT	ND

NT: Not Tested, ND: Not Determined.

SI= IC₅₀ for L929 cell line / IC₅₀ for A549 cell line.

After 24 h incubation, the apoptotic effects of compounds **1, 10, 11** and cisplatin on A549 cells were analyzed based on Annexin V-Propidium iodide (PI) binding capacities in flow cytometry. The apoptotic effects of compounds **1, 10, 11** and cisplatin on A549 cell line (at IC₅₀ values) were determined as 67.0, 64.8, 56.7 and 70.3% respectively (Table 2 and Figure 2). It can be concluded that these agents significantly induce apoptosis in A549 cells.

Table 2. Percents of typical quadrant analysis of Annexin V FITC/PI flow cytometry of A549 cells treated with compounds **1, 10, 11** and cisplatin.

Groups	Early apoptotic cells%	Late apoptotic cells%	Viable cells%	Necrotic cells%
Control	4.8	4.6	88.1	2.5
Cells treated with compound 1	4.4	62.6	8.7	24.3
Cells treated with compound 10	3.4	61.4	9.7	25.5
Cells treated with compound 11	3.2	53.5	12.3	31.1
Cells treated with cisplatin	2.8	67.5	8.0	21.7

A549 cells were cultured for 24 hours in medium with compounds **1, 10, 11** and cisplatin at IC₅₀ values. At least 10,000 cells were analyzed per sample, and quadrant analysis was performed.

After 24 h incubation of A549 cells with compounds **1, 10, 11**, and cisplatin (at IC₅₀ values), the effects of the compounds on mitochondrial membrane polarization and depolarization were investigated using quantitative mitochondrial membrane potential (MMP) assay by means of JC-1 staining followed by flow cytometry analysis (Table 3 and Figure 3). The mitochondrial membrane depolarized cell percentages caused by compounds **1, 10, 11** and cisplatin were determined as 64.8, 75.4, 75.9 and 55.4%, respectively. On the other hand, the mitochondrial membrane polarized cell percentages caused by these agents were found as 35.2, 23.7, 23.9 and 44.2%, respectively. According to these results, the effects of compounds **1, 10** and **11** on mitochondrial membrane depolarization were more significant than that of cisplatin.

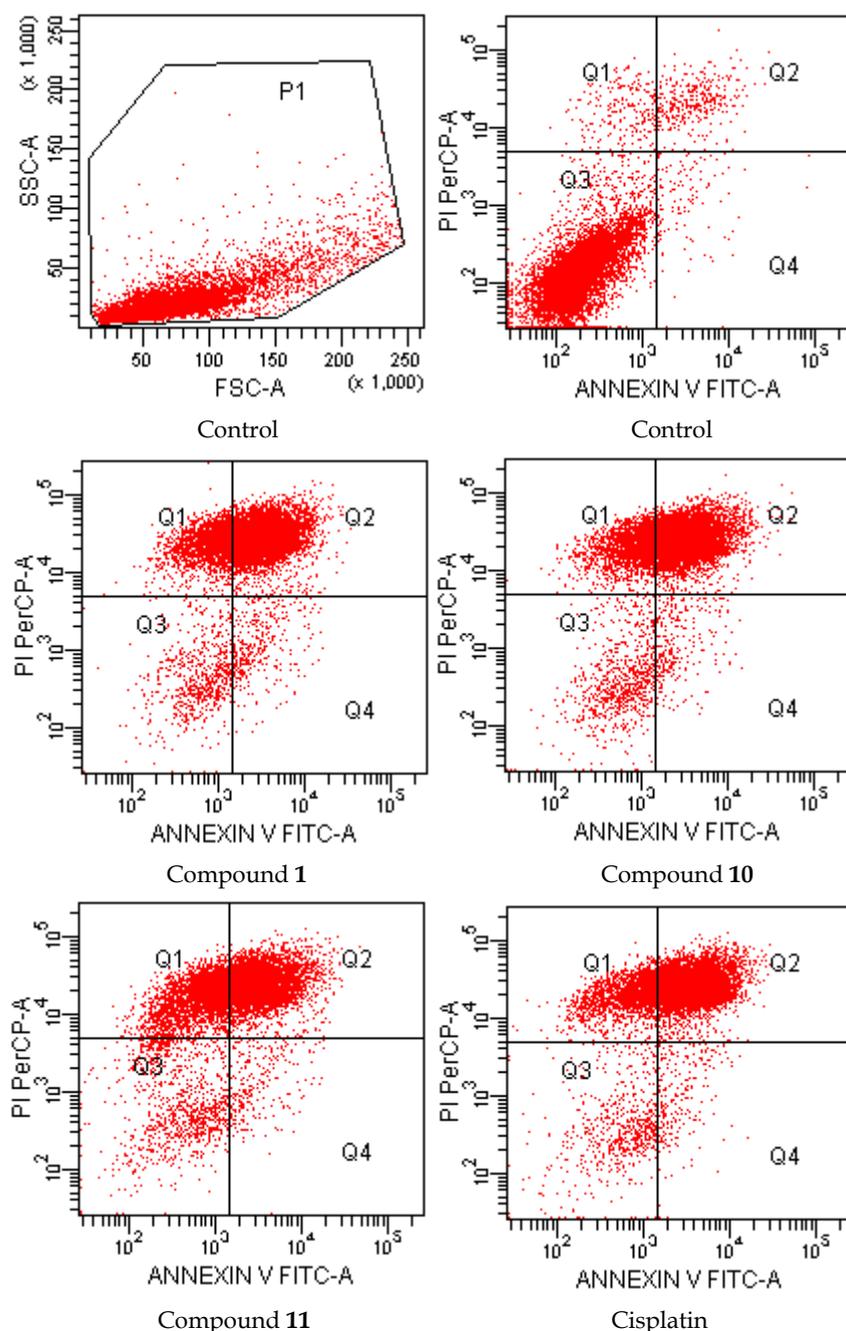


Figure 2. Flow cytometric analysis of A549 cells treated with IC₅₀ values of compounds **1**, **10**, **11** and cisplatin (14.33, 11.67, 16.67 and 18.33 µg/mL, respectively). At least 10,000 cells were analyzed per sample, and quadrant analysis was performed. Q1, Q2, Q3, and Q4 quadrants represent necrosis, late apoptosis, viable cells, and early apoptotic cells, respectively.

Table 3. The mitochondrial membrane polarized/depolarized percentages of A549 cells treated with compounds **1**, **10**, **11** and cisplatin.

Groups	P1 (%)	P2 (%)
Control	90.9	8.7
Cells treated with compound 1	35.2	64.8
Cells treated with compound 10	23.7	75.4
Cells treated with compound 11	23.9	75.9
Cells treated with cisplatin	44.2	55.4

P1: Mitochondrial membrane polarized cells, P2: Mitochondrial membrane depolarized cells.

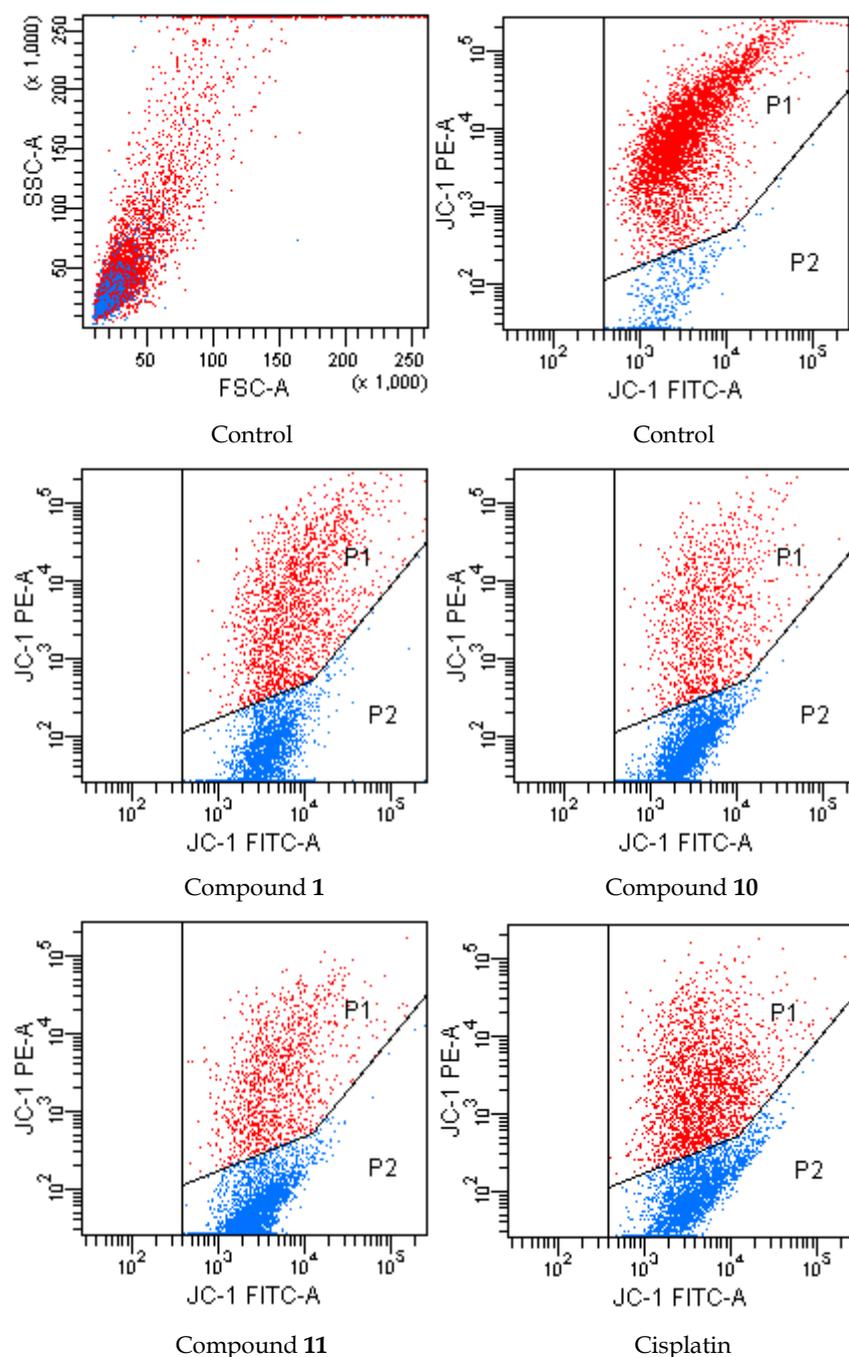


Figure 3. Flow cytometric analysis of mitochondrial membrane polarized / depolarized effects of compounds **1**, **10**, **11** and cisplatin on A549 cells (14.33, 11.67, 16.67 and 18.33 $\mu\text{g}/\text{mL}$, respectively). P1: mitochondrial membrane polarized cells, P2: mitochondrial membrane depolarized cells.

In order to explore the mechanism of action, compounds **1**, **10**, **11** and cisplatin (14.33, 11.67, 16.67 and 18.33 $\mu\text{g}/\text{mL}$, respectively) were evaluated for their ability to inhibit COX-1 and COX-2 in A549 cells after 24 h incubation (Table 4). 4-Chloro substituted compound **1** was found to be a non-selective COX inhibitor (69.01% for COX-1 and 58.74% for COX-2). On the other hand, compound **10**, which carry 2,5-dichlorophenyl at the 5th position of the furan ring on both sides, selectively inhibited COX-2 enzyme (6.96% for COX-1 and 54.81% for COX-2). According to the *in vitro* results, 2,5-dichloro substitution significantly enhanced selectivity for COX-2.

Table 4. The COX activities (U/mL) and the inhibitory effects of compounds **1**, **10**, **11** and cisplatin (at IC₅₀ values) on COXs in A549 cells after 24 h incubation.

	COX-1 activity (U/mL)	COX-2 activity (U/mL)	COX-1 inhibition%	COX-2 inhibition%
Control	4.09	4.29	NT	NT
Compound 1	1.27	1.78	69.01	58.74
Compound 10	3.81	1.94	6.96	54.81
Compound 11	3.36	2.96	17.81	31.23
Cisplatin	3.06	2.35	25.15	44.25
COX-1 standard	10.31	NT	NT	NT

NT: Not Tested.

3. CONCLUSION

In this paper, we described the synthesis of new bis(thiosemicarbazone) derivatives and evaluated their cytotoxic effects on A549 and L929 cell lines. Compounds **1**, **10** and **11** were identified as potent anticancer agents on A549 cell line. These compounds significantly induced apoptosis and mitochondrial membrane depolarization in A549 cells. Compound **10** was also found to be a selective COX-2 inhibitor, whilst compound **1** was found as a non-selective COX inhibitor. The *in vitro* studies clearly indicated that compound **10** exerted marked cytotoxic and apoptotic effects on A549 cell line through selective COX-2 inhibition.

4. MATERIALS AND METHODS

4.1. Chemistry

All reagents purchased from commercial suppliers were used without further purification. The Electrothermal IA9200 digital melting point apparatus (Staffordshire, UK) was used to determine the melting points (M.p.) of the compounds. IR spectra were recorded on an IRPrestige-21 Fourier Transform Infrared spectrophotometer (Shimadzu, Tokyo, Japan). ¹H NMR and ¹³C NMR spectra were recorded on a Varian Mercury-400 FT-NMR spectrometer (Agilent, Palo Alto, CA, USA). MS spectra were recorded on a VG Quattro Mass spectrometer (Agilent, Minnesota, USA). Elemental analyses were performed on a Perkin Elmer EAL 240 elemental analyzer (Perkin-Elmer, Norwalk, CT, USA).

4.1.1. General procedure for the synthesis of the compounds

1,4-Phenylenebis(thiosemicarbazide)

A mixture of *p*-phenylene diisothiocyanate (0.1 mol) and hydrazine hydrate (0.2 mol) in ethanol (30 mL) was stirred at room temperature for 4 h. Upon completion of the reaction, the precipitate was collected by filtration. The product was crystallized from ethanol [20].

N,N'-(1,4-phenylene)bis(2-((5-arylfuran-2-yl)methylene)hydrazine-1-carbothioamide) derivatives (**1-11**)

A mixture of 1,4-phenylenebis(thiosemicarbazide) (0.01 mol) and 5-arylfurfural (0.01 mol) was refluxed in ethanol for 8 h. Upon completion of the reaction, the mixture was cooled and the precipitate was collected by filtration. The product was crystallized from ethanol [20].

N,N'-(1,4-phenylene)bis(2-((5-(4-chlorophenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (**1**)

M.p. 244-245 °C. Yield 88%. IR ν_{\max} (cm⁻¹): 3282.84 (N-H stretching), 3126.61, 3020.53 (Aromatic C-H stretching), 2974.23 (Aliphatic C-H stretching), 1589.34, 1541.12, 1525.69, 1504.48, 1471.69 (N-H bending, C=N and C=C stretching), 1409.96, 1388.75, 1317.38, 1300.02, 1251.80, 1222.87, 1195.87, 1091.71, 1056.99, 1024.20, 1010.70 (C-H bending, C-N, C-O stretching and aromatic C-H in plane bending), 920.05, 827.46, 792.74, 719.45, 659.66, 624.94 (Aromatic C-H out of plane bending and C-S stretching). ¹H NMR (400 MHz, DMSO-*d*₆): 7.21 (q, *J* = 4.00, 6.40 Hz, 4H), 7.52 (d, *J* = 8.40 Hz, 4H), 7.62 (s, 4H), 7.88 (d, *J* = 8.80 Hz, 4H), 8.14 (s, 2H), 9.98 (s, 2H), 11.94 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): 109.07 (2CH), 115.72 (2CH), 125.27 (4CH), 125.65 (4CH), 128.28 (2C), 128.95 (4CH), 132.21 (2C), 132.60 (2C), 136.09 (2CH), 149.24 (2C), 153.57 (2C), 175.64 (2C). MS (FAB) *m/z* 634.06 [M+H]⁺. Anal. Calcd. for C₃₀H₂₂Cl₂N₆O₂S₂: C, 56.87; H, 3.50; N, 13.26. Found: C, 56.86; H, 3.51; N, 13.26.

N,N'-(1,4-phenylene)bis(2-((5-(3-chlorophenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (2)

M.p. 225-226 °C. Yield 84%. IR ν_{\max} (cm⁻¹): 3327.21, 3294.42 (N-H stretching), 3126.61, 3010.88 (Aromatic C-H stretching), 2972.31 (Aliphatic C-H stretching), 1597.06, 1544.98, 1519.91, 1490.97 (N-H bending, C=N and C=C stretching), 1411.89, 1381.03, 1261.45, 1205.51, 1193.94, 1080.14, 1022.27 (C-H bending, C-N, C-O stretching and aromatic C-H in plane bending), 981.77, 935.48, 910.40, 806.25, 769.60, 754.17, 682.80, 671.23 (Aromatic C-H out of plane bending and C-S stretching). ¹H NMR (400 MHz, DMSO-*d*₆): 7.26 (dd, *J* = 3.60, 18.80 Hz, 4H), 7.39 (d, *J* = 7.60 Hz, 2H), 7.49 (t, *J* = 8.00, 16.00 Hz, 2H), 7.65 (s, 4H), 7.83 (d, *J* = 8.00 Hz, 2H), 7.92 (s, 2H), 8.16 (s, 2H), 10.02 (s, 2H), 11.97 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): 109.75 (2CH), 115.56 (2CH), 122.461 (CH), 123.46 (CH), 125.25 (4CH), 127.80 (2CH), 130.75 (2CH), 131.38 (2CH), 132.18 (3C), 133.86 (3C), 136.09 (2CH), 149.54 (2C), 153.08 (2C), 175.66 (2C). MS (FAB) *m/z* 634.07 [M+H]⁺. Anal. Calcd. for C₃₀H₂₂Cl₂N₆O₂S₂: C, 56.87; H, 3.50; N, 13.26. Found: C, 56.85; H, 3.50; N, 13.28.

N,N'-(1,4-phenylene)bis(2-((5-(2-chlorophenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (3)

M.p. 227-228 °C. Yield 81%. IR ν_{\max} (cm⁻¹): 3329.14, 3296.35 (N-H stretching), 3122.75, 3053.32, 3014.74 (Aromatic C-H stretching), 2966.52 (Aliphatic C-H stretching), 1537.27, 1519.91, 1500.62, 1465.90 (N-H bending, C=N and C=C stretching), 1409.96, 1384.89, 1313.52, 1257.59, 1195.87, 1078.21, 1018.41 (C-H bending, C-N, C-O stretching and aromatic C-H in plane bending), 908.47, 835.18, 792.74, 756.10, 725.23, 613.36 (Aromatic C-H out of plane bending and C-S stretching). ¹H NMR (400 MHz, DMSO-*d*₆): 7.28 (d, *J* = 3.60 Hz, 2H), 7.34 (d, *J* = 4.00, 2H), 7.38 (td, *J* = 1.60, 7.60, 15.20 Hz, 2H), 7.49 (t, *J* = 7.60, 7.00, 14.60 Hz, 2H), 7.58 (d, *J* = 8.40 Hz, 2H), 7.64 (s, 4H), 8.06 (d, *J* = 7.60 Hz, 2H), 8.18 (s, 2H), 10.01 (s, 2H), 11.99 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): 113.35 (2CH), 114.93 (2CH), 125.23 (4CH), 127.57 (2CH), 127.62 (2CH), 128.24 (2CH), 129.26 (2CH), 129.35 (2C), 130.74 (2C), 132.15 (2C), 136.07 (2CH), 149.17 (2C), 150.74 (2C), 175.70 (2C). MS (FAB) *m/z* 634.06 [M+H]⁺. Anal. Calcd. for C₃₀H₂₂Cl₂N₆O₂S₂: C, 56.87; H, 3.50; N, 13.26. Found: C, 56.89; H, 3.49; N, 13.25.

N,N'-(1,4-phenylene)bis(2-((5-(4-nitrophenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (4)

M.p. 264-265 °C. Yield 91%. IR ν_{\max} (cm⁻¹): 3284.77 (N-H stretching), 3105.39, 3055.24, 3010.88 (Aromatic C-H stretching), 2953.02 (Aliphatic C-H stretching), 1597.06, 1537.27, 1519.91, 1489.05 (N-H bending, NO₂, C=N and C=C stretching), 1332.81, 1294.24, 1273.02, 1257.59, 1199.07, 1080.14, 1060.85, 1026.13 (C-H bending, NO₂, C-N, C-O stretching and aromatic C-H in plane bending), 981.77, 923.90, 852.54, 798.53, 763.81, 752.24, 692.44, 650.01 (Aromatic C-H out of plane bending and C-S stretching). ¹H NMR (400 MHz, DMSO-*d*₆): 7.31 (d, *J* = 4.00 Hz, 2H), 7.49 (d, *J* = 4.00 Hz, 2H), 7.61 (s, 4H), 8.09 (d, *J* = 9.20 Hz, 4H), 8.14 (s, 2H), 8.30 (d, *J* = 9.20 Hz, 4H), 10.01 (s, 2H), 12.00 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): 112.55 (2CH), 115.65 (2CH), 124.37 (4CH), 124.58 (4CH), 125.39 (4CH), 131.80 (2C), 135.13 (2C), 136.13 (2CH), 146.24 (2C), 150.98 (2C), 152.35 (2C), 175.83 (2C). MS (FAB) *m/z* 655.11 [M+H]⁺. Anal. Calcd. for C₃₀H₂₂N₈O₆S₂: C, 55.04; H, 3.39; N, 17.12. Found: C, 55.05; H, 3.39; N, 17.11.

N,N'-(1,4-phenylene)bis(2-((5-(2-nitrophenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (5)

M.p. 229-230 °C. Yield 87%. IR ν_{\max} (cm⁻¹): 3300.20 (N-H stretching), 3132.40 (Aromatic C-H stretching), 2974.23 (Aliphatic C-H stretching), 1597.06, 1516.05, 1504.48, 1473.62 (N-H bending, NO₂, C=N and C=C stretching), 1409.96, 1388.75, 1373.32, 1317.38, 1255.66, 1220.94, 1197.79, 1085.92, 1029.99, 1018.41 (C-H bending, NO₂, C-N, C-O stretching and aromatic C-H in plane bending), 923.90, 850.61, 788.89, 769.60, 746.45, 721.38, 704.02, 646.15, 630.72 (Aromatic C-H out of plane bending and C-S stretching). ¹H NMR (400 MHz, DMSO-*d*₆): 7.10 (d, *J* = 3.60 Hz, 2H), 7.22 (d, *J* = 3.60 Hz, 2H), 7.58 (t, *J* = 7.60, 15.20 Hz, 2H), 7.73 (t, *J* = 7.20, 9.60, 16.80 Hz, 6H), 7.92 (dd, *J* = 7.20, 19.20 Hz, 4H), 8.05 (s, 2H), 9.85 (s, 2H), 12.02 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): 112.09 (2CH), 114.80 (2CH), 121.71 (2CH), 123.81 (2CH), 124.39 (4CH), 128.54 (2CH), 129.53 (2CH), 131.41 (2C), 132.25 (2C), 135.74 (2CH), 146.92 (2C), 149.22 (2C), 150.43 (2C), 175.34 (2C). MS (FAB) *m/z* 655.12 [M+H]⁺. Anal. Calcd. for C₃₀H₂₂N₈O₆S₂: C, 55.04; H, 3.39; N, 17.12. Found: C, 55.02; H, 3.40; N, 17.13.

N,N'-(1,4-phenylene)bis(2-((5-(4-bromophenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (6)

M.p. 246-247 °C. Yield 85%. IR ν_{\max} (cm⁻¹): 3286.70 (N-H stretching), 3126.61, 3016.67 (Aromatic C-H stretching), 2980.02 (Aliphatic C-H stretching), 1579.70, 1539.20, 1525.69, 1502.55, 1467.83 (N-H bending, C=N and C=C stretching), 1409.96, 1388.75, 1317.38, 1280.73, 1251.80, 1222.87, 1193.94, 1087.85, 1055.06, 1024.20, 1006.84 (C-H bending, C-N, C-O stretching and aromatic C-H in plane bending), 918.12, 833.25, 810.10, 785.03, 715.59, 655.80, 621.08 (Aromatic C-H out of plane bending and C-S stretching). ¹H NMR (400 MHz, DMSO-*d*₆): 7.21 (s, 4H), 7.61 (s, 4H), 7.65 (d, *J* = 8.40 Hz, 4H), 7.81 (d, *J* = 8.80 Hz, 4H), 8.12 (s, 2H), 9.96 (s, 2H), 11.92 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): 112.06 (2CH), 115.73 (2CH), 121.20 (2C), 125.28 (4CH), 125.89 (4CH), 128.62 (4CH), 131.86 (2C), 132.21 (2C), 136.10 (2CH), 149.28 (2C), 153.61 (2C), 175.66 (2C). MS (FAB) *m/z* 723.96 [M+H]⁺. Anal. Calcd. for C₃₀H₂₂Br₂N₆O₂S₂: C, 49.87; H, 3.07; N, 11.63. Found: C, 49.89; H, 3.06; N, 11.62.

N,N'-(1,4-phenylene)bis(2-((5-(3-trifluoromethylphenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (7)

M.p. 226-227 °C. Yield 80%. IR ν_{\max} (cm⁻¹): 3334.92, 3294.42 (N-H stretching), 3130.47, 3012.81 (Aromatic C-H stretching), 2972.31 (Aliphatic C-H stretching), 1620.21, 1597.06, 1548.84, 1519.91, 1494.83, 1452.40 (N-H bending, C=N and C=C stretching), 1433.11, 1411.89, 1382.96, 1330.88, 1255.66, 1209.37, 1161.15, 1111.00, 1074.35, 1024.20 (C-H bending, C-N, C-O stretching and aromatic C-H in plane bending), 981.77, 937.40, 912.33, 894.97, 835.18, 779.24, 765.74, 694.37, 650.01 (Aromatic C-H out of plane bending and C-S stretching). ¹H NMR (400 MHz, DMSO-*d*₆): 7.28 (d, *J* = 3.60 Hz, 2H), 7.41 (d, *J* = 4.00 Hz, 2H), 7.66 (s, 4H), 7.72 (t, *J* = 7.20, 7.60, 14.80 Hz, 4H), 8.17 (s, 2H), 8.18 (s, 4H), 10.05 (s, 2H), 12.00 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): 110.13 (2CH), 115.46 (2CH), 120.29 (2CH), 122.63 (2CH), 124.38 (2C), 125.19 (2CH), 125.34 (2CH), 127.64 (2CH), 129.75 (2CH), 130.09 (2C), 130.42 (2C), 132.13 (2C), 136.11 (2CH), 149.82 (2C), 153.00 (2C), 175.67 (2C). MS (FAB) *m/z* 701.12 [M+H]⁺. Anal. Calcd. for C₃₂H₂₂F₆N₆O₂S₂: C, 54.85; H, 3.16; N, 11.99. Found: C, 54.84; H, 3.17; N, 11.97.

N,N'-(1,4-phenylene)bis(2-((5-(3,4-dichlorophenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (8)

M.p. 242-243 °C. Yield 86%. IR ν_{\max} (cm⁻¹): 3284.77 (N-H stretching), 3126.61, 3016.67 (Aromatic C-H stretching), 2974.23 (Aliphatic C-H stretching), 1537.27, 1523.76, 1502.55, 1454.33 (N-H bending, C=N and C=C stretching), 1409.96, 1386.82, 1319.31, 1255.66, 1193.94, 1136.07, 1112.93, 1087.85, 1066.64, 1024.20 (C-H bending, C-N, C-O stretching and aromatic C-H in plane bending), 933.55, 866.04, 833.25, 792.74, 719.45, 677.01, 626.87 (Aromatic C-H out of plane bending and C-S stretching). ¹H NMR (400 MHz, DMSO-*d*₆): 7.23 (d, *J* = 3.20 Hz, 2H), 7.32 (d, *J* = 3.60 Hz, 2H), 7.62 (s, 4H), 7.70 (d, *J* = 8.40 Hz, 2H), 7.83 (d, *J* = 8.40 Hz, 2H), 8.10 (s, 2H), 8.13 (s, 2H), 10.02 (s, 2H), 11.98 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): 110.34 (2CH), 115.67 (2CH), 123.92 (2CH), 125.36 (2CH), 125.45 (2CH), 129.92 (2CH), 130.29 (2CH), 131.13 (2C), 131.93 (2C), 132.00 (4C), 136.10 (2CH), 149.79 (2C), 152.15 (2C), 175.70 (2C). MS (FAB) *m/z* 703.98 [M+H]⁺. Anal. Calcd. for C₃₀H₂₀Cl₄N₆O₂S₂: C, 51.30; H, 2.87; N, 11.96. Found: C, 51.33; H, 2.86; N, 11.95.

N,N'-(1,4-phenylene)bis(2-((5-(2,4-dichlorophenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (9)

M.p. 249-250 °C. Yield 85%. IR ν_{\max} (cm⁻¹): 3275.13 (N-H stretching), 3132.40, 3012.81 (Aromatic C-H stretching), 2976.16 (Aliphatic C-H stretching), 1577.77, 1541.12, 1519.91, 1500.62, 1460.11 (N-H bending, C=N and C=C stretching), 1409.96, 1392.61, 1381.03, 1354.03, 1319.31, 1257.59, 1222.87, 1195.87, 1111.00, 1093.64, 1076.28, 1026.13 (C-H bending, C-N, C-O stretching and aromatic C-H in plane bending), 921.97, 867.97, 835.18, 817.82, 788.89, 719.45, 667.37, 626.87 (Aromatic C-H out of plane bending and C-S stretching). ¹H NMR (400 MHz, DMSO-*d*₆): 7.27 (d, *J* = 3.60 Hz, 2H), 7.37 (d, *J* = 4.00 Hz, 2H), 7.55-7.58 (m, 6H), 7.75 (s, 2H), 8.06 (d, *J* = 8.40 Hz, 2H), 8.13 (s, 2H), 10.00 (s, 2H), 11.97 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): 114.34 (2CH), 115.53 (2CH), 125.88 (2CH), 127.12 (2CH), 128.41 (2CH), 129.84 (2CH), 130.48 (2CH), 130.72 (2C), 132.45 (2C), 133.42 (4C), 136.63 (2CH), 149.98 (2C), 150.23 (2C), 176.29 (2C). MS (FAB) *m/z* 703.97 [M+H]⁺. Anal. Calcd. for C₃₀H₂₀Cl₄N₆O₂S₂: C, 51.30; H, 2.87; N, 11.96. Found: C, 51.31; H, 2.86; N, 11.97.

N,N'-(1,4-phenylene)bis(2-((5-(2,5-dichlorophenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (10)

M.p. 222-223 °C. Yield 83%. IR ν_{\max} (cm⁻¹): 3304.06 (N-H stretching), 3130.47, 3057.17 (Aromatic C-H stretching), 2976.16 (Aliphatic C-H stretching), 1585.49, 1552.70, 1487.12, 1465.90 (N-H bending, C=N and C=C stretching), 1398.39, 1379.10, 1350.17, 1317.38, 1292.31, 1244.09, 1220.94, 1174.65, 1099.43, 1026.13 (C-H bending, C-N, C-O stretching and aromatic C-H in plane bending), 997.20, 933.55, 904.61, 883.40, 829.39, 785.03, 709.80, 667.37, 613.36 (Aromatic C-H out of plane bending and C-S stretching). ¹H NMR (400 MHz, DMSO-*d*₆): 7.27 (s, 2H), 7.37 (m, 4H), 7.57 (d, *J* = 7.20 Hz, 6H), 8.01 (s, 2H), 8.11 (s, 2H), 10.06 (s, 2H), 11.98 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): 114.47 (2CH), 114.83 (2CH), 125.28 (2CH), 127.25 (2CH), 127.76 (2CH), 128.96 (2CH), 129.14 (2CH), 131.88 (2C), 132.42 (3C), 132.56 (3C), 136.12 (2CH), 149.26 (2C), 149.89 (2C), 175.75 (2C). MS (FAB) *m/z* 703.95 [M+H]⁺. Anal. Calcd. for C₃₀H₂₀Cl₄N₆O₂S₂: C, 51.30; H, 2.87; N, 11.96. Found: C, 51.29; H, 2.88; N, 11.96.

N,N'-(1,4-phenylene)bis(2-((5-(4-chloro-2-nitrophenyl)furan-2-yl)methylene)hydrazine-1-carbothioamide) (11)

M.p. 228-229 °C. Yield 82%. IR ν_{\max} (cm⁻¹): 3317.56 (N-H stretching), 3140.11, 3012.81 (Aromatic C-H stretching), 2974.23 (Aliphatic C-H stretching), 1589.34, 1521.84, 1500.62, 1473.62 (N-H bending, NO₂, C=N and C=C stretching), 1411.89, 1392.61, 1361.74, 1317.38, 1257.59, 1197.79, 1118.71, 1082.07, 1024.20 (C-H bending, NO₂, C-N, C-O stretching and aromatic C-H in plane bending), 985.62, 916.19, 877.61, 821.68, 783.10, 763.81, 721.38, 667.37 (Aromatic C-H out of plane bending and C-S stretching). ¹H NMR (400 MHz, DMSO-*d*₆): 7.15 (d, *J* = 4.00 Hz, 2H), 7.22 (d, *J* = 4.00 Hz, 2H), 7.70 (s, 4H), 7.80 (d, *J* = 8.60 Hz, 2H), 7.98 (d, *J* = 8.40 Hz, 2H), 8.03 (s, 2H), 8.14 (s, 2H), 9.84 (s, 2H), 12.07 (s, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): 112.75 (2CH), 114.96 (2CH), 120.33 (2CH), 123.79 (2CH), 124.43 (4CH), 129.75 (2CH), 131.19 (2C), 132.17 (2C), 133.37 (2C), 135.76 (2CH), 146.95 (2C), 148.13 (2C), 150.72 (2C), 175.34 (2C). MS (FAB) *m/z* 724.03 [M+H]⁺. Anal. Calcd. for C₃₀H₂₀Cl₂N₈O₆S₂: C, 49.80; H, 2.79; N, 15.49. Found: C, 49.82; H, 2.78; N, 15.48.

4.2. Biochemistry

4.2.1. Cell culture and drug treatment

A549 Human lung adenocarcinoma (ATCC® CCL-185™) and L929 mouse fibroblast (ATCC® CRL-6364™) cell lines were cultured and drug treatments were carried out as previously described [20].

4.2.2. MTT assay

MTT assay was performed as previously described in the literature [26] with small modifications [20]. Cisplatin was used as a positive control. Selectivity index (SI) values were also calculated according to the formula [27] below [Eq. 1]:

$$\text{SI} = \text{IC}_{50} \text{ for L929 cell line} / \text{IC}_{50} \text{ for A549 cell line} \quad [\text{Eq. 1}]$$

4.2.3. Flow cytometric analyses of apoptosis

After the incubation of A549 cells with compounds **1**, **10**, **11** and cisplatin at IC_{50} concentrations, phosphatidylserine externalization, which indicates early apoptosis, was detected using FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA) on a BD FACSAria flow cytometer for 24 h. Annexin V staining protocol was applied according to the manufacturer's instructions (BD Pharmingen, San Jose, CA, USA) and analyzed by a BD FACSAria flow cytometer using FACSDiva version 6.1.1 software (BD Biosciences, San Jose, CA, USA) [20].

4.2.4. Analysis of MMP by flow cytometry

A549 cells were seeded in six-well plates at a density of 10^5 cells/mL, and the IC_{50} concentration of compounds or cisplatin was added to cells. The cells were incubated in 5% CO_2 air-conditioned atmosphere at 37 °C. After 24 h of incubation, the cells were trypsinized, washed with PBS, and centrifuged at $400 \times g$ for 5 min. 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye solution (1 \times assay buffer + JC-1 stock solution) was added to the cells. The stock solution was prepared by dissolving DMSO. Then the samples were incubated at a temperature of 37 °C for 10-15 min. After incubation, the cells were washed twice with an assay buffer and analyzed by BD FACSAria flow cytometry using BD FACSDiva software version 6.1.1 (BD Biosciences, San Jose, CA, USA). The cells showing mitochondrial membrane potential disruption were determined as a percentage of all cells [28].

4.2.5. In vitro COX inhibition assay

The lysates of A549 cells which were incubated with IC_{50} concentrations of the compounds for 24 hours were obtained and used in COX assay. COX activity assay kit (Cayman Chemical, Ann Arbor, MI, USA) was used for the determination of COX activity in A549 cell line. Briefly, 150 μL of all samples was incubated in boiling water for 5 min. and centrifuged at $8000 \times g$ for 1 min. Then supernatants were used for obtaining background values. 120 μL assay solutions, 10 μL hemin and 40 μL sample and inactive samples were administered in triplicates for sample and background wells. 110 μL assay solution, 10 μL hemin and 40 μL sample and 10 μL DuP-697 or SC-560 were added (DuP-697 inhibits COX-2 activity, whereas SC-560 inhibits COX-1 activity) to inhibitor wells. The plate was shaken and incubated at 25 °C for 5 min. 20 μL colorimetric substrat was added to all wells. Then, 20 μL arachidonic acid solution was added and the reaction was started. The plate was shaken and incubated at 25 °C for 5 min. Then absorbance values were recorded at 540 nm.

4.2.6. Statistical analyses

Statistical Package for the Social Sciences (SPSS) for Windows 15.0 was used for statistical analysis. Data was expressed as Mean \pm SD. Comparisons were performed by one way ANOVA test for normally distributed continuous variables and post hoc analyses of group differences were expressed by the Tukey test.

Acknowledgements: This study was supported by Anadolu University Scientific Research Projects Commission under the grant no: 1905S052.

Author contributions: Concept – B.S., A.Ö., M.D.A.; Design – B.S., A.Ö., M.D.A.; Supervision – A.Ö., M.D.A.; Resources – B.S., A.Ö., M.D.A.; Materials – B.S., G.A.Ç., M.D.A.; Data Collection and/or Processing – B.S., G.A.Ç., M.D.A.; Analysis and/or Interpretation – B.S., G.A.Ç., M.D.A.; Literature Search – B.S., M.D.A.; Writing – B.S., G.A.Ç., M.D.A.; Critical Reviews – B.S., G.A.Ç., A.Ö., M.D.A.

Conflict of interest statement: The authors declared no conflict of interest.

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