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FULL PAPER

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Novel indole hydrazide derivatives: Synthesis and their antiproliferative activities through inducing apoptosis and DNA damage

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Abstract

A series of novel indole hydrazide derivatives was synthesized and evaluated for their anticancer activities. Compound **12** exhibited the highest antiproliferative activity against the MCF-7 cell line, with an IC_{50} value of 3.01 μM. Treatment of MCF-7 cells with compound **12** led to cell cycle arrest at the G0/G1 phase and also displayed a significant annexin V binding pattern, indicating that compound **12** is effective in apoptotic cell death. The Western blot analysis showed that compound **12** increased the expression of proapoptotic Bax and decreased the levels of the antiapoptotic Bcl-2 protein. It was also observed that MCF-7 cells treated with compound **12** showed reduced levels of procaspase-3 and -9 proteins. Moreover, compound **12** treatment induced a significant DNA damage in MCF-7 cells by increasing H2AX and ATM phosphorylation.

KEYWORDS

anticancer activity, apoptosis, cell cycle, DNA damage, synthesis

1 | INTRODUCTION

The indole scaffold is a privileged structure in medicinal chemistry due to its versatile pharmacological activities such as antibacterial, antifungal, anti-inflammatory, antioxidant, antiviral, and anticancer activities. In the last decade, numerous indole derivatives have been reported to demonstrate anticancer activities through different targets.[1] Camalexin (Figure 1), an indole phytoalexin, was reported as a potent antitumor agent against human leukemia cell lines and primary human AML cells through the reactive oxygen species (ROS)–endoplasmic reticulum (ER) stress–mitochondrial apoptosis pathway.[2] Zhang et al. reported 5-chloro-3-phenyl-indole-2-carboxylic acid (4-nitrobenzylidene)-hydrazide (**I**) (Figure 1) as a potent apoptosis inducer through cell-based high-throughput screening assay. Compound **I** showed caspase activation with an EC_{50} value of 0.1 μM in T47D breast cancer cells and growth inhibition with a GI_{50} value of 0.9 μM in T47D cells.[3] The Spallarossa group synthesized indole-based analogs, which were evaluated for their antiproliferative activity against leukemia, breast, and renal cancer cell lines. Among them, compound **II** (Figure 1) showed strong proapoptotic potential and a partial depolymerizing effect on tubulin.[4] El-Nakkady et al.[5] reported a series of 2-phenylindole derivatives as potential anticancer agents. Compound **III** (Figure 1) exhibited a remarkable antitumor activity, with an IC_{50} value of 1.6 nM, against the MCF-7 breast carcinoma cell line. Moreover, a series of indole-2-carbohydrazide derivatives was synthesized, with some of them displaying potent cytotoxic activities against HCT116 and SW480 cell lines with GI_{50} values in sub-micromolar concentrations.[6] Haddach et al.[7] identified 3-(5-((2-oxoindolin-3-ylidene)methyl)furan-2-yl)amide derivative (CX-6258) (Figure 1) as a pan-Pim kinase inhibitor with excellent antiproliferative potency in cellular assays. More recently, panobinostat (Figure 1) has been approved as an oral pan-histone deacetylase inhibitor for the treatment of relapsed multiple myeloma. It can also inhibit tumor growth and

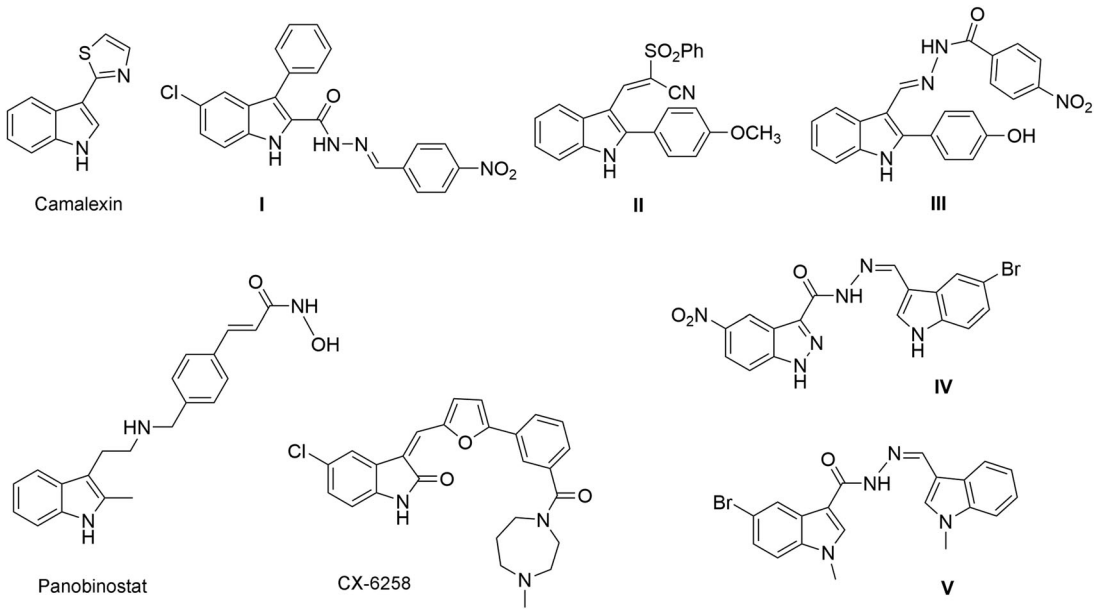


FIGURE 1 Chemical structures of some indole derivatives showing anticancer activity

metastasis.[8,9] Sreenivasulu et al. reported the synthesis and anticancer evaluation of a series of hydrazide–hydrazone-linked indole and indazole moieties, and *N*-methyl and *N*,*N*-dimethyl bis (indolyl)hydrazide–hydrazone derivatives.[10,11] Among them, compound **IV**[10] exhibited strong cytotoxicity, with an IC_{50} value of 1.93 μM, against the MCF-7 breast cancer cell line, as compared with the standard drug doxorubicin (IC_{50} value: 0.98 μM). Compound **V**[11] induced apoptosis via a caspase-independent pathway through the participation of mitogen-activated protein kinases such as extracellular signal-regulated kinase and p38 as well as p53 pathways.

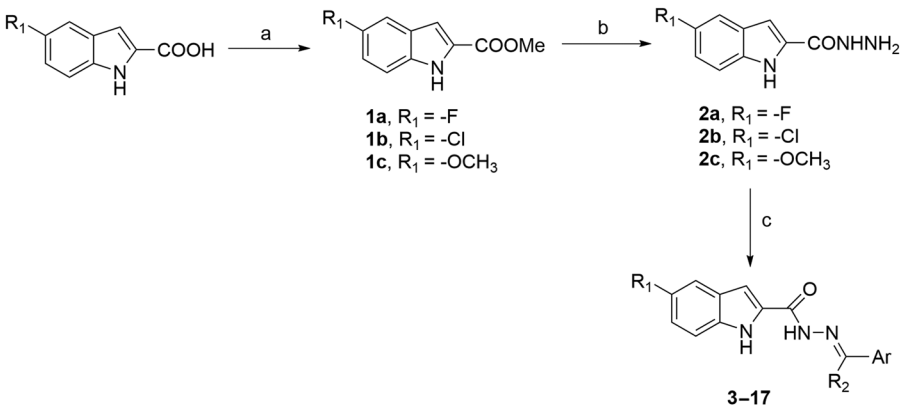
In light of the abovementioned observations regarding indole compounds, it is understood that substitution on 2-, 3-, and 5-positions of the indole scaffold is useful for anticancer activity. Therefore, in this study, we synthesized a series of 5-substituted indole hydrazide derivatives and evaluated their anticancer activities.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

The general methods for preparing indole-2-carbohydrazide derivatives are shown in Scheme 1. First, the methyl esters of indole-2-carboxylic acid derivatives (**1a–c**) were prepared in a quantitative yield.[12] Hydrazide derivatives of indoles (**2a–c**) were obtained by the reaction of indole esters with hydrazine hydrate in ethanol.[13] Hydrazide derivatives were reacted with appropriate aromatic aldehydes or ketones in the presence of the catalytic amount of glacial acetic acid[14] to afford the target compounds (**3–17**).

It is well known that *N*-acylhydrazones may exist as geometrical isomers (*E*/*Z*) and as *cis*/*trans* rotamers due to the assemblage of imine functions (C=N) and amide bond (CO–NH), respectively. The acylhydrazones of aromatic aldehydes are reported to be prevailingly or completely in the *E*-configuration.[15–17] In ^{1}H nuclear magnetic



SCHEME 1 The synthesis method for the target compounds. Reagents and conditions: (a) H_2SO_4, methanol, reflux, overnight; (b) hydrazine hydrate, methanol, reflux; (c) appropriate aldehyde or ketone derivatives, absolute ethanol, glacial acetic acid, reflux

resonance (NMR) spectra of aldehyde-derived compounds, one set of singlet belongs to N=CH and CO–NH protons, indicating the presence of a single isomer of the compounds. The appearance of duplicate signals for amide and methyl protons in ^{1}H NMR spectra of compounds (**7** and **16**) derived from ketone revealed that these compounds exist as a mixture of two possible rotamers or *E*/*Z* isomers. Unlike ketone derivatives **7** and **16**, in ^{1}H NMR spectrum of compound **11**, one set of singlet arising from imine and amide protons was determined.

2.2 | Biological activity

2.2.1 | Antiproliferative activity

Antiproliferative activities of all synthesized compounds (**3**–**17**) were evaluated against MCF-7, HepG2, and PC3 cell lines. Initially, the cancer cells were treated with compounds **3**–**17** at 20-μM concentration. The cell viability % (CV%) values were calculated after treating cells for 48 hr. The CV% results are presented in Table 1, and it has been shown that all compounds generally have better antiproliferative activity against MCF-7 than HepG2 and PC3 cell lines. Compounds **5**, **12**, and **14** demonstrated higher cytotoxicity than other compounds against MCF-7 cells. However, almost all compounds demonstrated moderate, low, or no antiproliferative activity against HepG2 and PC3 cell lines.

Meanwhile, as shown in Figure 2, the three active compounds (**5**, **12**, and **14**) significantly decreased the cell viability in a dose-dependent manner ($p < .01$). The IC_{50} values of compounds **5**, **12**, and **14** were determined as 7.32, 3.01, and 5.48 μM, respectively, for 48 hr in MCF-7 cells. Regarding the activity against MCF-7 cells, 5-methoxyindole derivatives (**12**–**17**) were found to be more potent than the fluoro (**3**–**8**) and chloro (**9**–**11**) derivatives. Replacement of the 3,4-dichlorobenzylidene moiety (**14**) with 4-chlorobenzylidene (**12**) increased the anticancer activity. However, the five-ring scaffold such as pyrrole (**8**, **10**, and **17**) or imidazole (**9**) instead of benzene ring at the side chain of the indole scaffold decreased the anticancer activity. Structure–activity relationship results suggested that a methoxy group at the 5-position of the indole ring and chloro substitution on the benzylidene moiety are important for the anticancer activity.

2.2.2 | Cell cycle assay

The cell cycle assay was performed for further evaluation of the cytotoxic activity of compound **12** against MCF-7 cells. The cells were treated with IC_{50} concentration of compound **12** for 48 hr. As shown in Figure 3, the cell population percent at the G0/G1 phase increased to 45.6 ± 1.13% in compound **12**-treated group, whereas it was 1.7 ± 0.14% in the control group, suggesting that compound **12** induced a significant cell cycle arrest at the G0/G1 phase ($p < .0001$).

2.2.3 | Apoptosis assay

According to the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) assay results, compound **12** showing the strongest antiproliferative activity against MCF-7 cells has been selected for further assays. The apoptotic effects of compound **12** on MCF-7 cells were evaluated using the annexin V binding analyses through flow cytometry. Treatment of MCF-7 cells with IC_{50} value of compound **12** for 48 hr significantly induced apoptosis, as shown in Figure 4. The percentage of the early apoptotic and late apoptotic cells in compound **12**-treated MCF-7 cells increased to 29.13 ± 2.99% and 27.18 ± 0.99%, respectively, compared with the control cells (2.49 ± 1.50% and 1.50 ± 0.70%, respectively; $p < .01$).

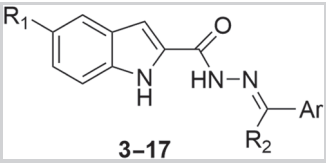
2.2.4 | DNA damage analysis

To demonstrate whether compound **12** treatment induced DNA damage in MCF-7 cells, the cells were treated with an IC_{50} concentration of compound **12** for 48 hr and then DNA damage assay was performed. Treatment of MCF-7 cells with compound **12** significantly induced DNA damage response in MCF-7 cells, as shown in Figure 5. After compound **12** exposure, the level of H2AX and ATM phosphorylation markedly increased as compared with control cells. The percentage of DNA double-strand breaks and total single-strand breaks (pATM) in compound **12**-treated cells increased to 24.12 ± 2.19% and 38.44 ± 2.30%, respectively, compared with the control cells (5.40 ± 1.40% and 6.8 ± 1.69%, respectively; $p < .01$).

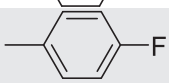
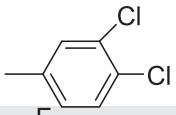
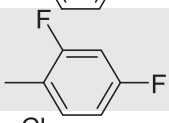
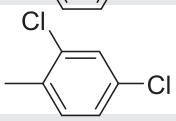
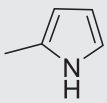
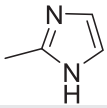
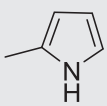
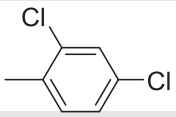
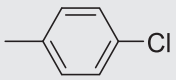
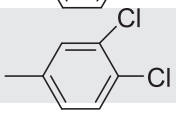
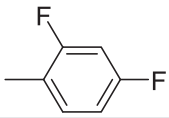
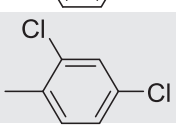
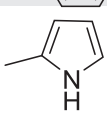
2.2.5 | Western blot analysis

The Western blot analysis was performed to evaluate the alteration in the expression of apoptosis-related proteins. In this context, the proapoptotic Bax and antiapoptotic Bcl-2 protein expression levels were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Figure 6, after the treatment of MCF-7 cells with compound **12**, the proapoptotic Bax expression significantly increased as compared with untreated control ($p < .05$), whereas there was a decrease in the expression of antiapoptotic Bcl-2. Although the apoptotic potential of compound **12** has been demonstrated by annexin V binding analysis, its effect on the levels of proapoptotic caspases has also been evaluated. Caspases are a group of endoprotease enzymes that hydrolyze peptide bonds in a reaction that depends on catalytic cysteine residues in the caspase and aspartic acid residues in the substrate.[18] Initially, caspases are produced as inactive monomeric procaspases, and they become active after a dimerization or cleavage process.[19] In the present study, we evaluated the alteration in procaspase-3 and -9 expression after the treatment of MCF-7 cells with compound **12**. The results showed that caspase-3 expression significantly reduced as compared with control ($p < .01$), confirming the caspase-activating effect of compound **12**.

TABLE 1 The percentage cell viability (CV%) of the cancer cell lines after 48 hr of exposure to synthesized compounds at 20 μM



3–17

Compounds	R_1	R_2	Ar	MCF-7	HepG2	PC3
3	F	H		42.04 ± 2.13	78.86 ± 3.45	71.47 ± 1.18
4	F	H		99.84 ± 3.12	98.19 ± 4.17	101 ± 2.16
5	F	H		**15.09 ± 1.37** (7.32 μM)	49.81 ± 3.79	40.63 ± 3.26
6	F	H		49.66 ± 2.88	66.30 ± 3.11	71.49 ± 1.08
7	F	CH_3		53.92 ± 1.13	60.88 ± 4.56	74.19 ± 1.17
8	F	H		107.95 ± 1.49	79.56 ± 1.09	69.34 ± 2.07
9	Cl	H		58.41 ± 2.93	92.63 ± 4.13	98.32 ± 2.54
10	Cl	H		68.72 ± 4.19	73.22 ± 2.79	62.1 ± 1.56
11	Cl	CH_3		31.97 ± 1.29	70.65 ± 1.13	64.61 ± 4.12
12	OCH_3	H		**23.03 ± 3.51** (3.01 μM)	63.64 ± 1.11	62.11 ± 4.02
13	OCH_3	H		38.45 ± 3.13	68.38 ± 2.31	78.9 ± 1.32
14	OCH_3	H		**22.15 ± 4.59** (5.48 μM)	67.54 ± 3.09	50.76 ± 1.04
15	OCH_3	H		25.50 ± 1.09	62.09 ± 1.13	54.71 ± 3.19
16	OCH_3	CH_3		26.15 ± 1.91	54.52 ± 5.47	46.13 ± 3.12
17	OCH_3	H		42.86 ± 4.01	69.57 ± 4.57	53.13 ± 2.18

Note: Data are presented as mean ± standard deviation. The three most active compounds are represented in bold.

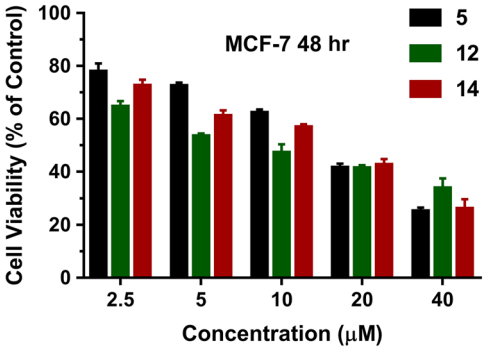


FIGURE 2 The antiproliferative activity of compounds **5**, **12**, and **14** against the most sensitive cell line MCF-7. The cells were treated with the compounds at different concentrations (2.5–40 μM) for 48 hr, and the cell viability was assessed using the XTT assay. All data are expressed as mean ± *SD* of three replicates. All the cell viability values at 2.5-, 5-, 10-, 20-, and 40-μM concentrations are significantly different from control ($p < .01$). *SD*, standard deviation; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide

3 | CONCLUSION

In this study, we synthesized a series of novel 5-substituted indole hydrazide derivatives and evaluated their anticancer activities. Compound **12** exhibited the highest cytotoxic activity against MCF-7 human breast cancer cells with IC_{50} value of 3.01 μM. Exposure of MCF-7 cells to compound **12** significantly induced apoptosis. Preliminary mechanism of action studies showed that compound **12** increased the expression of proapoptotic Bax and decreased the expression of antiapoptotic Bcl-2 protein. Also, treatment of MCF-7 cells with compound **12** resulted in reduced levels of procaspase-3 and -9 proteins, indicating that compound **12** induced apoptosis through a caspase-dependent pathway. Moreover, in compound **12**-treated experiments, the level of H2AX and ATM phosphorylation, the percentage of DNA double-strand breaks, and total single-strand breaks increased, confirming DNA damage effect of compound **12**.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

Commercial reagents were used without further purification. The NMR spectra (400 MHz) were recorded on a Varian Mercury 400 NMR spectrometer (Varian Inc., Palo Alto, CA) using tetramethylsilane as the internal standard. Thin-layer chromatography was performed on F_{254} (Merck) silica gel plates. The electrospray ionization (ESI) spectra were recorded on a Waters ZQ Micromass liquid chromatography–mass spectrometry (LC–MS) spectrometer (Waters Corporation, Milford, MA) with ESI source as ionization. The elemental analysis was performed using a Leco-932 CHNS-O Analyzer (Leco, St. Joseph, MI) within ±0.4% of the theoretical values.

The original spectra of the investigated compounds as well as their InChI codes, together with some biological activity data, are provided as Supporting Information Data.

4.1.2 | General procedure for the synthesis of methyl esters of indole-2-carboxylic acids 1a–c

Indole-2-carboxylic acids (1 mmol) were refluxed in MeOH (5 ml) in the presence of concentrated sulfuric acid (0.1 ml) overnight. Then, the mixture was concentrated and saturated $NaHCO_3$ (aq) was added. The precipitate was filtered, washed with water, and dried to afford the target compounds (**1a**,**b**,[20] **1c**[21]).

4.1.3 | General procedure for the synthesis of indole-2-carbohydrazide derivatives 2a–c

Hydrazine hydrate (10 mmol) was added to a suspension of methyl esters of indole-2-carboxylic acids (**1a**–**c**, 1 mmol) in methanol (15 ml).

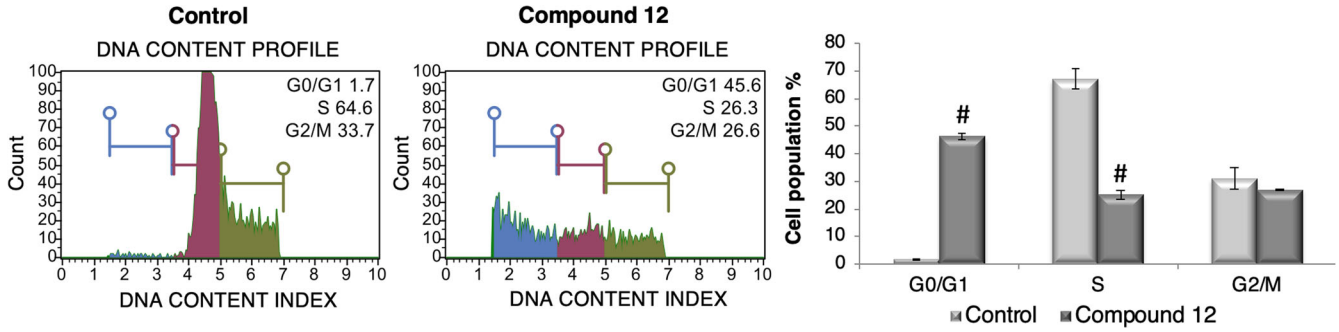


FIGURE 3 The effect of compound **12** on cell cycle inhibition. The MCF-7 cells were treated with compound **12** with an IC_{50} value of 3.01 μM for 48 hr, and the cell population was detected by Muse Cell Analyzer (Millipore, Germany). The nontreated cells were used as control. The results indicate mean ± standard deviation of two independent experiments. The difference is expressed as $^{\#}p < .0001$, compared with control

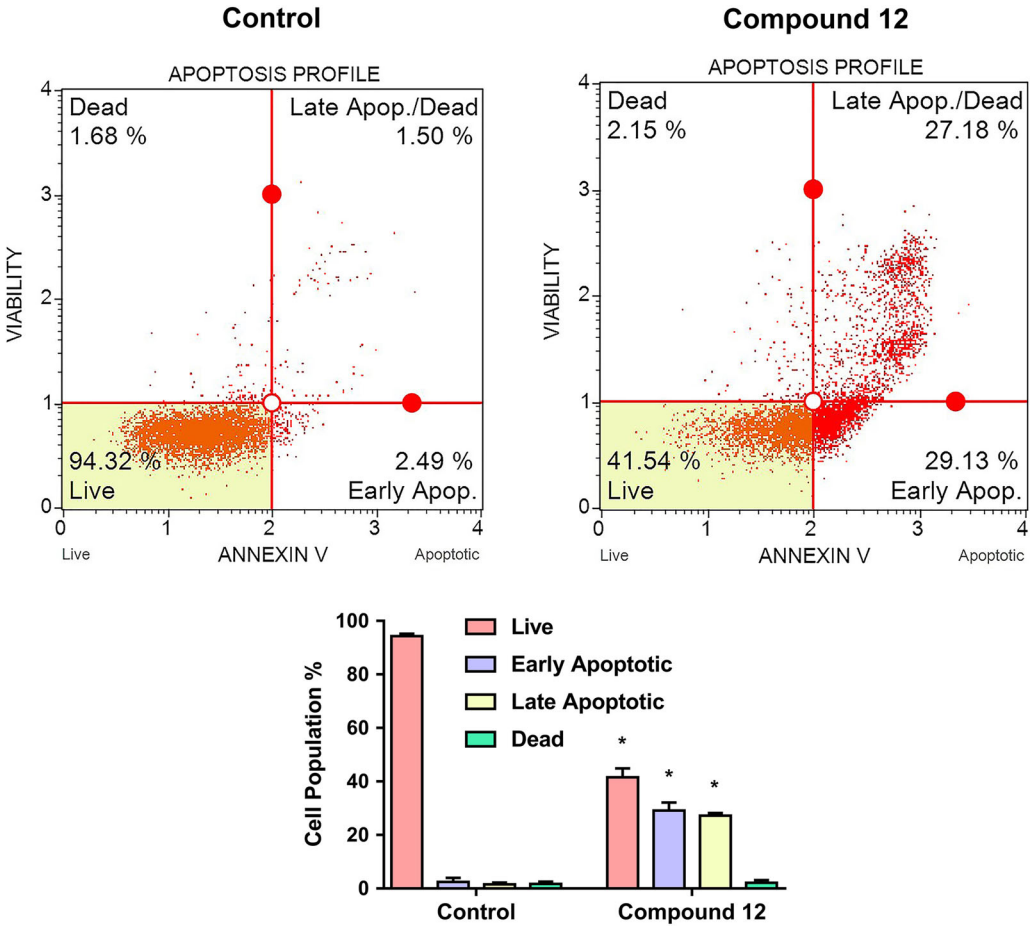


FIGURE 4 Apoptotic effects of compound **12** on MCF-7 cells. The cells were treated with the IC_{50} concentration of compound **12** and incubated for 48 hr, and the apoptosis was detected by Muse Cell Analyzer (Millipore). All experiments were carried out in triplicate, thereby obtaining similar results. Statistically significant differences are expressed as $^{*}p < .01$, compared with the control

The reaction mixture was refluxed for 3 hr, cooled to room temperature, and filtered to afford hydrazide derivatives (**2a**,[22] **2b**,[6] **2c**[13]).

4.1.4 | General procedures for the synthesis of compounds 3–17

Indole-2-carbohydrazide derivatives (**2a**–**c**, 1 mmol), appropriate aromatic aldehyde or ketone derivatives (1 mmol), and the catalytic amount of glacial acetic acid in absolute ethyl alcohol were refluxed for 24 hr. The precipitate was filtered and washed with ethanol. The crude products were recrystallized from ethanol to afford the pure target compounds **3**–**17**.

N′-(4-Chlorobenzylidene)-5-fluoro-1H-indole-2-carbohydrazide ***(3)***

Yield: 93%; Mp: 263–265°C. ^{1}H NMR (400 MHz, dimethyl sulfoxide [DMSO]-d_6) δ: 7.09 (td, 1H, J = 8.8 Hz, 2.4 Hz), 7.31 (s, 1H, H-3), 7.44–7.49 (m, 2H), 7.53 (d, 2H, J = 8.4 Hz), 7.78 (d, 2H, J = 8.4 Hz), 8.44 (s, 1H, –N=CH), 11.94 (s, 1H, NH), 12.01 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 103.59, 105.84, 112.62, 113.54, 126.94, 128.76, 131.55, 133.16, 133.52, 134.40, 145.91, 155.99, 157.30, 158.31. MS (ESI) m/z: 316.08 [M + H]. Anal. calcd. for $C_{16}H_{11}ClFN_3O{\cdot}0.2H_2O$ (%): C, 60.17; H, 3.59; N, 13.15. Found (%): C, 59.92; H, 3.58; N, 13.87.

5-Fluoro-N′-(4-fluorobenzylidene)-1H-indole-2-carbohydrazide ***(4)***

Yield: 70%; Mp: 251–253°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.087 (td, 1H, J = 13.6 Hz, 2.4 Hz), 7.29 (t, 3H), 7.43–7.47 (m, 2H), 7.80 (t, 2H), 8.44 (s, 1H, –N=CH), 11.92 (s, 2H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 103.55, 105.89, 112.626, 113.594, 115.90, 127.03, 129.26, 130.87, 131.71, 133.56, 146.22, 156.06, 157.34, 158.38, 161.86, 164.32. MS (ESI) m/z: 300.52 [M + H]. Anal. calcd. for $C_{16}H_{11}F_2N_3O$ (%): C, 64.21; H, 3.70; N, 14.04. Found (%): C, 64.18; H, 3.71; N, 14.25.

*N′-(3,4-Dichlorobenzylidene)-5-fluoro-1*H*-indole-2-carbohydrazide* ***(5)***

Yield: 68%; Mp: 257–260°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.10 (t, 1H), 7.33 (s, 1H), 7.45–7.48 (m, 2H), 7.70–7.77 (m, 2H), 7.97 (d, 1H, J = 1.6 Hz), 8.42 (s, 1H), 11.92 (s, 1H, NH), 12.10 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 103.89, 105.90, 112.75, 113.59, 126.87, 127.01, 128.47, 131.04, 131.69, 132.16, 133.60, 135.10, 144.52, 156.05, 158.36. MS (ESI) m/z: 350.22 [M + H]. Anal. calcd. for

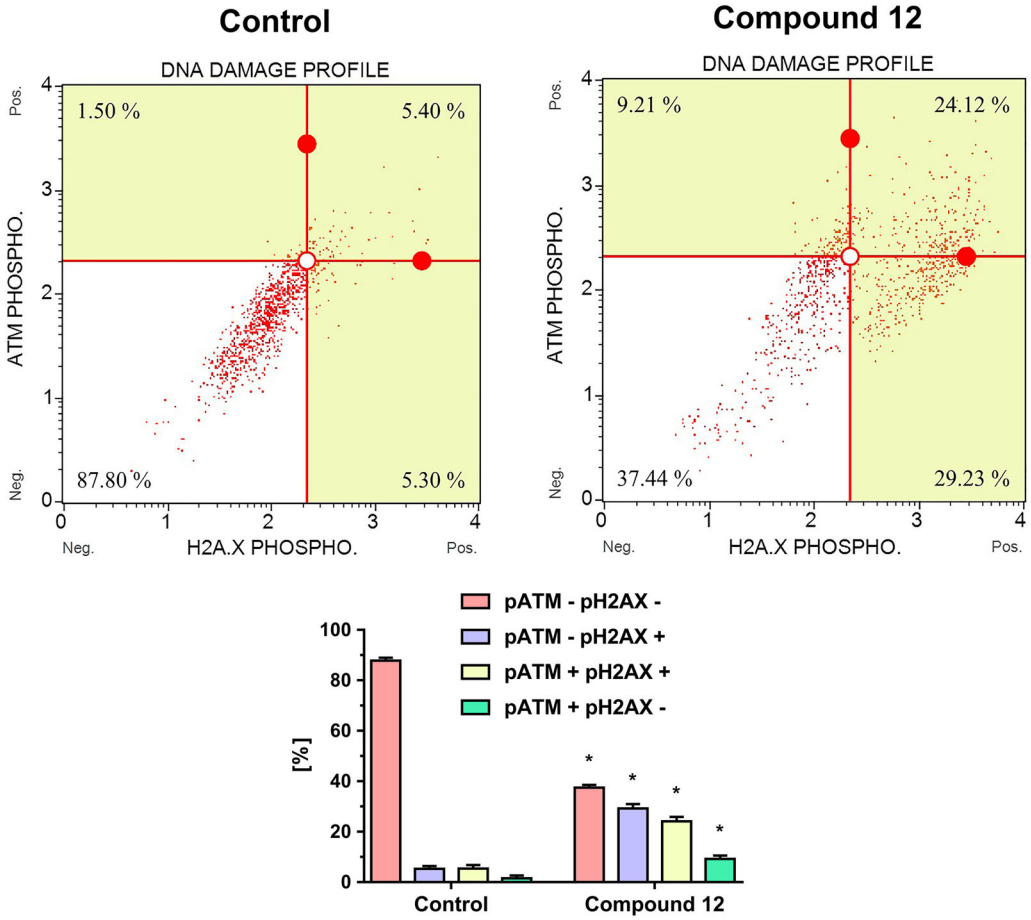


FIGURE 5 Compound **12** induced DNA damage response in MCF-7 cells. The cells were treated with compound **12** at IC_{50} concentration for 48 hr to induce DNA damage, and activation of ATM and H2AX was determined using Muse™ Multi-Color DNA Damage Kit (Merck Millipore). Results are expressed as mean ± standard deviation; $^{*}p < .01$

$C_{16}H_{10}Cl_2FN_3O{\cdot}0.025H_2O$ (%): C, 54.80; H, 2.88; N, 11.98. Found (%): C, 54.41; H, 2.94; N, 12.30.

N′-(2,4-Difluorobenzylidene)-5-fluoro-1H-indole-2-carbohydrazide ***(6)***

Yield: 54%; Mp: 233–235°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.09 (t, 1H), 7.21 (s, 1H), 7.30–7.39 (m, 2H), 7.47 (d, 2H, J = 7.6 Hz), 8.01 (d, 1H, J = 3.2 Hz), 8.63 (s, 1H), 11.94 (s, 1H, NH), 12.05 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 103.99, 104.62, 105.974, 112.76, 118.76, 127.048, 127.97, 131.51, 133.66, 139.25, 156.09, 157.36, 158.41, 159.74, 162.03, 164.43. MS (ESI) m/z: 340.51 [M + Na]. Anal. calcd. for $C_{16}H_{10}F_3N_3O{\cdot}1.2H_2O$ (%): C, 56.70; H, 3.68; N, 12.39. Found (%): C, 56.47; H, 3.77; N, 12.65.

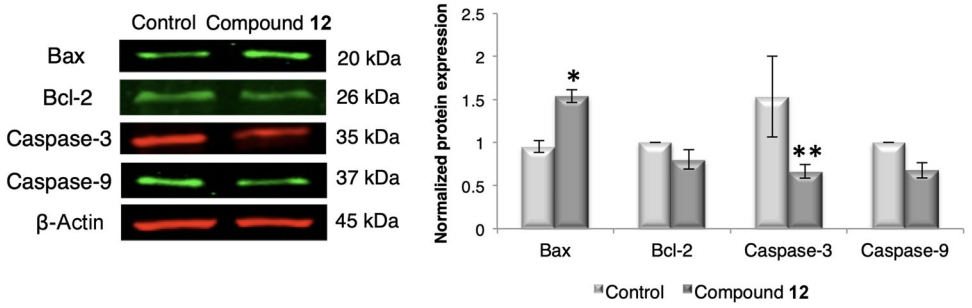


FIGURE 6 Effects of compound **12** on apoptotic protein expression in MCF-7 cells. The cells were treated with 3.01 μM of compound **12** for 48 hr, and total cell lysates were prepared and immunoblotted to detect Bax, Bcl-2, and caspase-3/-9. β-Actin served as a loading control. Results were expressed as the mean ± standard deviation; $^{*}p < .05$, $^{**}p < .01$, as compared with control

N′-[1-(2,4-Dichlorophenyl)ethylidene]-5-fluoro-1H-indole-2-carbohydrazide ***(7)***

Yield: 72%; Mp: 204–206°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.30 (s, 3H, CH_3), 7.03–7.11 (m, 1H), 7.37–7.47 (m, 2H), 7.52 (s, 1H), 7.55 (dd, 1H, J = 8.0 Hz, 2.0 Hz), 7.72 (t, 1H), 7.75 (d, 1H, J = 2.0 Hz), 10.30 (s, 1H, NH), 11.89 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 18.32, 105.94, 112.63, 113.56, 126.97, 127.56, 128.06, 129.35, 130.78, 131.59, 132.19, 133.28, 133.95, 134.62, 137.99, 155.92, 158.29. MS (ESI) *m/z*: 364.68 [M + H], 386.54 [M + Na]. Anal. calcd. for $C_{17}H_{12}Cl_2FN_3O$ (%): C, 56.06; H, 3.32; N, 11.53. Found (%): C, 55.77; H, 3.64; N, 11.62.

N′-[(1H-Pyrrol-2-yl)methylene]-5-fluoro-1H-indole-2-carbohydrazide ***(8)***

Yield: 50%; Mp: 243–245°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 6.13 (s, 1H), 6.49 (s, 1H), 6.90 (s, 1H), 7.04 (t, 1H), 7.23 (s, 1H), 7.40–7.44 (m, 2H), 8.27 (s, 1H, –N=CH), 11.52 (s, 1H, NH), 11.58 (s, 1H, NH), 11.86 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 102.91, 105.674, 109.20, 112.182, 113.374, 122.48, 126.89, 127.0, 132.09, 133.32, 140.47, 155.96, 156.77, 158.27. MS (ESI) *m/z*: 271.54 [M + H]. Anal. calcd. for $C_{14}H_{11}FN_4O{\cdot}0.3H_2O$ (%): C, 60.99; H, 4.24; N, 20.32. Found (%): C, 60.90; H, 4.08; N, 20.63.

N′-[(1H-Imidazol-2-yl)methylene]-5-chloro-1H-indole-2-carbohydrazide ***(9)***

Yield: 80%; Mp: 318–320°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.14–7.28 (m, 4H), 7.45 (d, 1H, J = 8.8 Hz), 7.76 (s, 1H), 8.36 (s, 1H, –N=CH), 12.25 (bs, 3H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 103.13, 113.92, 120.77, 123.91, 124.40, 127.95, 131.49, 135.17, 139.28, 142.34, 157.24. MS (ESI) *m/z*: 288.39 [M + H]. Anal. calcd. for $C_{13}H_{10}ClN_5O{\cdot}0.15H_2O$ (%): C, 53.76; H, 3.57; N, 24.11. Found (%): C, 53.50; H, 3.72; N, 23.86.

N′-[(1H-Pyrrol-2-yl)methylene]-5-chloro-1H-indole-2-carbohydrazide ***(10)***

Yield: 38%; Mp: 253–254°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 6.14 (s, 1H), 6.50 (s, 1H), 6.91 (s, 1H), 7.18–7.23 (m, 2H), 7.43 (d, 1H, J = 8.8 Hz), 7.74 (s, 1H), 8.27 (s, 1H, –N=CH), 11.54 (s, 1H, NH), 11.64 (s, 1H, NH), 11.98 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 102.54, 109.31, 113.46, 113.89, 120.68, 122.62, 123.66, 124.37, 126.97, 128.07, 131.97, 135.07, 140.61, 156.80. MS (ESI) *m/z*: 287.50 [M + H]. Anal. calcd. for $C_{14}H_{11}ClN_4O{\cdot}0.2H_2O$ (%): C, 57.91; H, 3.95; N, 19.29. Found (%): C, 57.70; H, 4.03; N, 19.61.

5-Chloro-N′-[1-(2,4-dichlorophenyl)ethylidene]-1H-indole-2-carbohydrazide ***(11)***

Yield: 90%; Mp: 216–218°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.36 (s, 3H, CH_3), 7.20 (dd, 1H, J = 8.8 Hz, 2.0 Hz), 7.35 (s, 1H), 7.43–7.50 (m, 3H), 7.70 (d, 2H, J = 1.2 Hz), 10.90 (s, 1H, NH), 11.95 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 18.32, 113.94, 120.88, 124.04, 124.34, 127.57, 127.97, 129.24, 131.60, 132.19, 133.95, 135.06, 137.97. MS (ESI) *m/z*: 402.43 [M + Na]. Anal. calcd. for $C_{17}H_{12}Cl_3N_3O{\cdot}0.07H_2O$ (%): C, 53.46; H, 3.20; N, 11.00. Found (%): C, 53.08; H, 3.22; N, 11.44.

N′-(4-Chlorobenzylidene)-5-methoxy-1H-indole-2-carbohydrazide ***(12)***

Yield: 84%; Mp: 243–245°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.75 (s, 3H, CH_3), 6.86 (dd, 1H, J = 9.2 Hz, 2.4 Hz), 7.12 (s, 1H), 7.21 (d, 1H, J = 3.2 Hz), 7.33 (d, 1H, J = 8.8 Hz), 7.51 (d, 2H, J = 8.0 Hz), 7.75 (d, 2H, J = 8.4 Hz), 8.42 (s, 1H), 11.66 (s, 1H, NH), 11.89 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 55.18, 102.01, 103.34, 113.14, 115.17, 127.21, 128.57, 128.85, 130.08, 132.11, 133.24, 134.28, 145.51, 153.80, 157.54. MS (ESI) *m/z*: 328.50 [M + H], 350.46 [M + 23]. Anal. calcd. for $C_{17}H_{14}ClN_3O_2{\cdot}0.02H_2O$ (%): C, 62.22; H, 4.31; N, 12.80. Found (%): C, 61.83; H, 4.40; N, 12.88.

N′-(4-Fluorobenzylidene)-5-methoxy-1H-indole-2-carbohydrazide ***(13)***

Yield: 75%; Mp: 213–215°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.76 (s, 3H, CH_3), 6.87 (dd, 1H, J = 9.2 Hz, 2.4 Hz), 7.13 (s, 1H), 7.22 (s, 1H), 7.28–7.36 (m, 3H), 7.78–7.82 (m, 2H), 8.45 (s, 1H), 11.65 (s, 1H, NH), 11.85 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 55.74, 102.56, 103.77, 113.69, 115.65, 116.38, 127.79, 129.66, 130.70, 131.44, 132.65, 146.30, 154.35, 158.08, 162.28, 164.74. MS (ESI) *m/z*: 312.61 [M + H], 334.55 [M + Na]. Anal. calcd. for $C_{17}H_{14}FN_3O_2{\cdot}0.05H_2O$ (%): C, 65.39; H, 4.55; N, 13.45. Found (%): C, 65.00; H, 4.83; N, 13.42.

N′-(3,4-Dichlorobenzylidene)-5-methoxy-1H-indole-2-carbohydrazide ***(14)***

Yield: 78%; Mp: 274–276°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.77 (s, 3H, CH_3), 6.89 (dd, 1H, J = 8.4 Hz, 2.4 Hz), 7.14 (s, 1H), 7.26 (s, 1H), 7.36 (d, 1H, J = 9.2 Hz), 7.70–7.76 (m, 2H), 7.96 (s, 1H), 8.41 (s, 1H), 11.69 (s, 1H, NH), 12.04 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 55.26, 102.09, 103.69, 113.26, 115.40, 126.81, 127.28, 128.43, 130.0, 131.08, 131.71, 132.09, 132.26, 135.24, 144.15, 153.91, 157.75. MS (ESI) *m/z*: 362.62 [M + H], 384.37 [M + Na]. Anal. calcd. for $C_{17}H_{13}Cl_2N_3O_2$ (%): C, 56.37; H, 3.61; N, 11.60. Found (%): C, 56.00; H, 3.75; N, 11.87.

N′-(2,4-Difluorobenzylidene)-5-methoxy-1H-indole-2-carbohydrazide ***(15)***

Yield: 70%; Mp: 245–247°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.77 (s, 3H, CH_3), 6.89 (dd, 1H, J = 9.0 Hz, 2.4 Hz), 7.14 (d, 1H, J = 2.4 Hz), 7.19–7.23 (m, 2H), 7.36 (d, 1H, J = 8.8 Hz), 7.40 (d, 1H, J = 2.0 Hz), 7.98–8.04 (m, 1H), 8.64 (s, 1H), 11.68 (s, 1H, NH), 11.97 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 55.26, 102.10, 103.44, 104.48, 112.63, 113.24, 115.33, 118.81, 127.30, 127.90, 130.09, 132.23, 138.79, 153.90, 157.65, 159.62, 162.51, 164.46. MS (ESI) *m/z*: 330.58 [M + H], 352.48 [M + Na]. Anal. calcd. for $C_{17}H_{13}F_2N_3O_2{\cdot}0.01H_2O$ (%): C, 61.97; H, 3.98; N, 12.75. Found (%): C, 61.65; H, 4.31; N, 12.71.

N′-[1-(2,4-Dichlorophenyl)ethylidene]-5-methoxy-1H-indole-2-carbohydrazide ***(16)***

Yield: 60%; Mp: 196–197°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.29 (s, 3H, CH_3), 3.72 (s, 3H, CH_3), 6.85 (td, 1H, J = 9.6 Hz, 2.4 Hz), 7.08 (s, 1H), 7.28–7.35 (m, 2H), 7.44 (d, 1H, J = 8.4 Hz), 7.51–7.56 (m, 1H), 7.74 (d, 1H, J = 15.2 Hz), 10.12 (s, 1H, NH), 11.52 (s, 1H, NH). ^{13}C

NMR (100 MHz, DMSO-d_6) δ: 24.31, 55.20, 102.02, 113.66, 115.17, 127.18, 127.58, 128.10, 129.36, 130.79, 131.61, 131.90, 132.20, 133.23, 133.90, 134.62, 138.07, 153.76. MS (ESI) *m/z*: 376.48 [M + H], 398.48 [M + Na]. Anal. calcd. for $C_{18}H_{15}Cl_2N_3O_2$ (%): C, 57.46; H, 4.01; N, 11.16. Found (%): C, 57.19; H, 4.37; N, 11.41.

*N′-[(1H-Pyrrol-2-yl)methylene]-5-methoxy-1H-indole-2-carbohydrazide (**17**)*

Yield: 55%; Mp: 126–128°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.74 (s, 3H, CH_3), 6.13 (s, 1H), 6.48 (s, 1H), 6.84 (d, 1H, J = 8.4 Hz), 6.90 (s, 1H), 7.10 (d, 1H, J = 2.0 Hz), 7.15 (s, 1H), 7.31 (d, 1H, J = 8.4 Hz), 8.27 (s, 1H), 11.51 (s, 2H, NH), 11.60 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 55.18, 101.95, 102.63, 109.17, 113.05, 113.17, 114.71, 122.38, 126.97, 127.27, 130.62, 131.92, 140.14, 153.75, 157.08. MS (ESI) *m/z*: 283.60 [M + H]. Anal. calcd. for $C_{15}H_{14}N_4O_2 \cdot 0.6H_2O \cdot 0.6C_2H_5OH$ (%): C, 60.66; H, 5.90; N, 17.46. Found (%): C, 60.38; H, 5.93; N, 17.59.

4.2 | Biological evaluation

4.2.1 | Cell culture

MCF-7 (HTB-22), human breast cancer cell line, PC3 (CRL-1435), human prostate cancer cell line, and HepG2 (HB-8065), human hepatocellular carcinoma cell line, were purchased from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin (Sigma-Aldrich). The cultured cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. All newly synthesized compounds were dissolved in DMSO, and stock solutions were diluted with DMEM as the final concentration of DMSO did not exceed 0.5%.

4.2.2 | Cell viability assay

Metabolically active cells were evaluated using the XTT colorimetric assay (Roche Diagnostic, Germany). The MCF-7, HepG2, and PC3 cells were plated in 96-well plates at a density of 1×10^4 cells/well in 100-µl regular culture media, and they were allowed to attach overnight before treatment. Then, the cells were treated with 20-µM constant concentration of the compounds to determine CV% for 48 hr. Additionally, the cells were treated with selected three compounds, which were found to be most effective against MCF-7 cells, at 2.5-, 5-, 10-, 20-, 40-µM final concentrations for 48 hr. After incubation, DMEM, which contained the compounds, was removed and the wells were washed twice with 200 µl phosphate-buffered saline (PBS). Afterward, 100 µl DMEM without phenol red and a mixture of 50 µl XTT labeling solution were added to each well, and then the plates were incubated at 37°C for 4 hr. The plates were shaken and the absorbances were measured using an Enzyme-Linked Immunosorbent Assay Microplate Reader (Thermo Fisher Scientific, Germany) at 450 nm. All experiments were performed in triplicate, and the cell viability was expressed as a viable cell amount percentage, compared with control as untreated cells. The IC_{50} values of compounds **15**, **12**, and **14** in MCF-7 cells were calculated by GraphPad Prism 7 software (GraphPad Software Inc.).

4.2.3 | Cell cycle assay

The cell cycle analysis was performed by Muse Cell Cycle Assay Kit (Merck Millipore, Germany). The MCF-7 cells were treated with 3.01 µM of compound **12** for 48 hr, and the nontreated cells were used as control. The cells were then fixed with ethanol and prepared for the analysis according to the instructions. The cell population at G0/G1, S, and G2/M stages of the cell cycle was measured by Muse Cell Analyzer (Millipore, Germany).

4.2.4 | Annexin V binding assay

MCF-7 cells were treated with IC_{50} concentration of compound **12**, which is obtained from XTT analysis, and the extent of apoptosis was assessed using the Muse Annexin V/Dead Cell (Merck Millipore) assay. Shortly before treatment, the cells were seeded in six-well plates and were allowed to attach overnight. The next day, the cells were treated with compound **12** at its IC_{50} concentration and incubated for 48 hr. After incubation, the cells were collected, diluted with PBS containing 1% FBS, and incubated with Annexin V and Dead Cell reagent for 20 min at room temperature in the dark. The events for live, dead, early, and late apoptotic cells were counted by Muse Cell Analyzer (Millipore).

4.2.5 | DNA damage assay

ATM and H2AX activation was assessed using the Muse Multi-Color DNA Damage Kit (Merck Millipore) according to the manufacturer's instructions. First, the cells were treated with the IC_{50} concentration of compound **12** and incubated for 48 hr at 37°C. The cells were then centrifuged, washed once with PBS, and resuspended in 1× assay buffer. Then, the cells were fixed in a fixation buffer and incubated for 10 min on ice. Next to fixation, the cells were washed and permeabilized in the ice-cold buffer. Then, 10 µl antibody working cocktail solution was added to each tube that contained the cell suspension and incubated for 30 min in the dark at room temperature. Finally, the cells were centrifuged and washed, and then the cells were resuspended in 200 µl 1× assay buffer, and percentages of negative cells (no DNA damage), percentages of ATM-activated cells, percentages of H2AX-activated cells, and percentages of DNA double-strand breaks were evaluated using Muse Multi-Color DNA Damage software module (Millipore).

4.2.6 | Western blot assay

The MCF-7 cells treated with compound **12** at 3.01-μM concentration were lysed on ice in lysis buffer with proteinase inhibitor cocktail (Cell Signal Technology, Germany). The protein concentration was measured by bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL), and 20 μg of denaturated protein samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After the transfer process, the membranes were incubated overnight at 4°C with primary antibodies (Bax, Bcl-2, procaspase-3, procaspase-9, 1:1,000 [CST, Germany]) diluted in 1× PBS. The membranes were then incubated with IRDye secondary antibody diluted (1:1,000) in 1× PBS with 0.1% Tween-20 for 1 hr. After the final wash, the membranes were visualized with the CLX System (LI-COR Biosciences) by using IR screening. The band intensity was quantified by densitometric analysis using Image Studio Lite software (LI-COR Biosciences). The values were normalized to β-actin, and these results were expressed as a fold change.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests.

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REFERENCES

[1] S. Dadashpour, S. Emami, *Eur. J. Med. Chem.* **2018**, *150*, 9.
[2] Y. Yang, G. Wang, W. Wu, S. Yao, X. Han, D. He, J. He, G. Zheng, Y. Zhao, Z. Cai, R. Yu, *Oxid. Med. Cell. Longevity* **2018**, *14*, 7426950.
[3] H. Z. Zhang, J. Drewe, B. Tseng, S. Kasibhatla, S. X. Cai, *Bioorg. Med. Chem.* **2004**, *12*, 3649.
[4] A. Spallarossa, C. Caneva, M. Caviglia, S. Alfei, S. Butini, G. Campiani, S. Gemma, M. Brindisi, D. M. Zisterer, S. A. Bright, C. D. Williams, E. Crespan, G. Maga, G. Sanna, I. Delogu, G. Collu, R. Loddo, *Eur. J. Med. Chem.* **2015**, *102*, 648.
[5] S. S. El-Nakkady, M. M. Hanna, H. M. Roaiah, I. A. Y. Ghannam, *J. Med. Chem.* **2012**, *47*, 387.
[6] J. Zhang, T. Liu, M. Chen, F. Liu, X. Liu, J. Zhang, J. Lin, Y. Jin, *ChemMedChem* **2018**, *13*, 1181.
[7] M. Haddach, J. Michaux, M. K. Schwaebe, F. Pierre, S. E. O'Brien, C. Borsan, J. Tran, N. Raffaele, S. Ravula, D. Drygin, A. Siddiqui-Jain, L. Darjania, R. Stansfield, C. Proffitt, D. Macalino, N. Streiner, J. Bliesath, M. Omori, J. P. Whitten, K. Anderes, W. G. Rice, D. M. Ryckman, *ACS Med. Chem. Lett.* **2012**, *3*, 135.
[8] N. Fortunati, F. Marano, A. Bandino, R. Frairia, M. G. Catalano, G. Boccuzzi, *Int. J. Oncol.* **2014**, *44*, 700.
[9] G. Qin, Y. Li, X. Xu, X. Wang, K. Zhang, Y. Tang, H. Qiu, D. Shi, C. Zhang, Q. Long, *Cell Signaling* **2019**, *59*, 62.
[10] R. Sreenivasulu, P. Sujitha, S. S. Jadav, M. J. Ahsan, C. G. Kumar, R. R. Raju, *Monatsh. Chem.* **2017**, *148*, 305.
[11] R. Sreenivasulu, K. T. Reddya, P. Sujithab, C. G. Kumarb, R. R. Raju, *Bioorg. Med. Chem.* **2019**, *27*, 1043.
[12] S. N. Mistry, J. Shonberg, C. J. Draper-Joyce, C. Klein Herenbrink, M. Michino, L. Shi, A. Christopoulos, B. Capuano, P. J. Scammells, J. R. Lane, *J. Med. Chem.* **2015**, *58*, 6819.
[13] M. S. Almutairi, A. S. Zakaria, P. P. Ignasius, R. I. Al-Wabli, I. H. Joe, M. I. Attia, *J. Mol. Struct.* **2018**, *1153*, 333.
[14] M. S. Baharudin, M. Taha, S. Imran, N. H. Ismail, F. Rahim, M. T. Javid, K. M. Khan, M. Ali, *Bioorg. Chem.* **2017**, *72*, 323.
[15] M. Zhou, Y. J. Eun, I. A. Guzei, D. B. Weibe, *ACS Med. Chem. Lett.* **2013**, *4*, 880.
[16] R. Y. Morjan, A. M. Mkadmh, I. Beadham, A. A. Elmanama, M. R. Mattar, J. Raftery, R. G. Pritchard, A. M. Awadallah, J. M. Gardiner, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 5796.
[17] A. Y. Ershova, I. V. Lagodab, S. I. Yakimovichc, V. V. Pakal'nisc, I. V. Zerovac, A. V. Dobrodumova, V. V. Shamanina, *Russ. J. Org. Chem.* **2009**, *45*, 660.
[18] D. R. Mcllwain, T. Berger, T. W. Mak, *Cold Spring Harbor Perspect. Biol.* **2013**, *5*, a008656.
[19] R. C. Taylor, S. P. Cullen, S. J. Martin, *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 231.
[20] Z. Zhu, J. Yuan, Y. Zhou, Y. Qin, J. Xu, Y. Peng, *Eur. J. Org. Chem.* **2014**, *2014*, 511.
[21] D. Coowar, J. Bouissac, M. Hanbali, M. Paschaki, E. Mohier, B. Luu, *J. Med. Chem.* **2004**, *47*, 6270.
[22] W. T. A. Harrison, H. S. Yathirajan, B. V. Ashalatha, K. K. V. Raj, B. Narayana, *Acta Crystallogr., Sect. E: Crystallogr. Commun.* **2006**, *E62*, o4986.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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