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RESEARCH PAPER

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Development of novel benzofuran-isatin conjugates as potential antiproliferative agents with apoptosis inducing mechanism in Colon cancer

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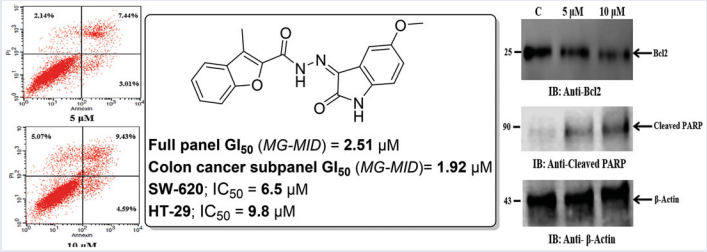
ABSTRACT

In the current work, a new set of carbohydrazide linked benzofuran-isatin conjugates (**5a–e** and **7a–i**) was designed and synthesised. The anticancer activity for compounds (**5b–d**, **7a, 7b, 7d** and **7g**) was measured against NCI-55 human cancer cell lines. Compound **5d** was the most efficient, and thus subjected to the five-dose screen where it showed excellent broad activity against almost all tested cancer subpanels. Furthermore, all conjugates (**5a–e** and **7a–i**) showed a good anti-proliferative activity towards colorectal cancer SW-620 and HT-29 cell lines, with an excellent inhibitory effect for compounds **5a** and **5d** (IC_{50} = 8.7 and 9.4 μM (**5a**), and 6.5 and 9.8 μM for (**5d**), respectively). Both compounds displayed selective cytotoxicity with good safety profile. In addition, both compounds provoked apoptosis in a dose dependent manner in SW-620 cells. Also, they significantly inhibited the anti-apoptotic Bcl2 protein expression and increased the cleaved PARP level that resulted in SW-620 cells apoptosis.

GRAPHICAL ABSTRACT



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1. Introduction

Cancer, a large family of diseases, is characterised by fast and uncontrolled cell division and differentiation mechanisms and has the potential to spread to or invade other body parts[1]. For several decades, cancer is considered one of the major world public health problems, and it remains a serious reason of the death of human beings all over the world[2]. Despite the presence of a variety of cancer treatment strategies, the majority of which induces non-selective cell death by targeting the DNA synthesis[3–6] and/or the replication machinery[7–10]. These early strategies are accompanied by severe side effects due to the unspecific cytotoxicity towards the cancer cells in addition to the resistance developed against them[4]. Therefore, the development of safe and effective novel anticancer agents with increased selective treatment strategies towards cancer cells has received more attention and still ongoing active search[11,12].

Recent strategies for anticancer development are to target specific biomarkers required for cancer cells division and/or induction of cell apoptosis such as deregulated, mutated, or over expressed proteins[13] and thus, affect cancer cells selectively with minimum influences on normal cells[14]. Among these targets are the anti-apoptotic protein Bcl2 and Poly ADP-ribose polymerase (PARP). In this regard, several reports stated that numerous of cancer cells are characterised by anti-apoptotic proteins (Bcl2) over-expression, which could lead to prevention of cell apoptosis as well as development of drug resistance[15,16]. On the other hand, PARP is a family of proteins involved in numerous cellular functions such as DNA repair and genomic stability[17–19] and also, PARP was

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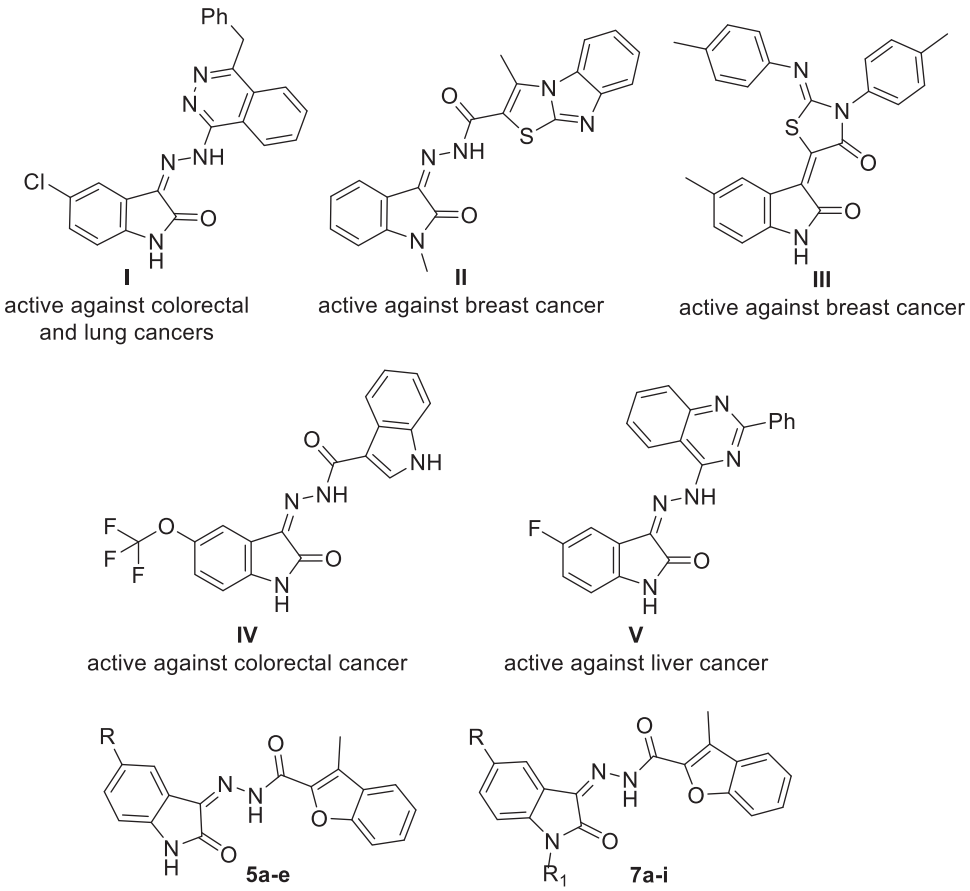


Figure 1. Structures of some reported isatin-bearing conjugates (**I–V**), as well as structures for target benzofuran-isatin conjugates (**5a–e** and **7a–i**).

reported to activate programmed cell death, through cleavage into PAR (Poly ADP-ribose), which motivates mitochondria to produce apoptosis inducing factors[20]. Thus, the development of compounds that inhibit the antiapoptotic Bcl2 proteins and/or potentiate the cleavage of PARP could be a promising approach to identify new anticancer therapies.

Heterocyclic compounds in particular oxygen containing heterocycles represent an important class of compounds possessing interesting pharmacological and biological activities[21–23]. Benzofuran nucleus, as a key functional scaffold, represents a basic structure in a diversity of biologically active synthetic and natural products[24–26], with broad range of desirable activities including; anti-Alzheimer's[27], antibacterial[28], anti-tubercular[29], antioxidant[30], anti-inflammatory[31], as well as antitumor activities[32]. Benzofuran derivatives exert their antiproliferative activity with diversified mechanisms such as inhibition of tubulin polymerisation[33,34], HIF-1[35], Aurora B kinase[36] and VEGFR-2 activity[37]. Furthermore, some benzofurans mediate their antiproliferative activity via apoptosis induction in various human cancer cell lines[38–40]. In addition, benzofuran-based conjugates were largely studied and were found to exert significant anticancer activity, such as conjugation of benzofuran with pyrazole[41], indole[42] and others[43,44].

On the other hand, isatin is identified as a privileged nucleus that included in many pharmacologically active small molecules, such as antiviral[45], antimicrobial[46], anticonvulsant[47], CNS-acting[48], as well as anticancer[49,50] agents. Over the last few years, hybridisation of isatin nucleus with different heterocycles has been reported as a successful approach to develop efficient antitumor agents towards different cancer types through diverse enzymatic and cellular mechanisms[49,50]. To name just a few, isatin-phthalazine (compound **I**)[51], isatin-thiazolo[3,2-*a*]benzimidazole (compound **II**)[52], isatin-thiazolidinone (compound **III**)[53], isatin-indole (compound **IV**)[54] and isatin-quinazoline (compound **V**)[55] conjugates were reported to possess promising anticancer activities (Figure 1).

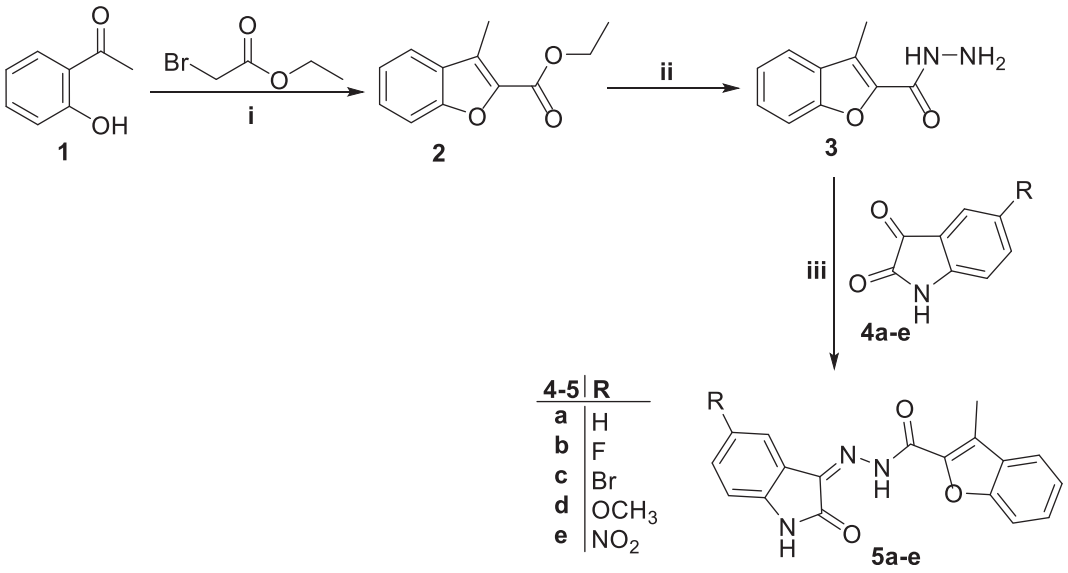
Encouraged by the aforementioned findings and considering the need to develop safe and effective novel anticancer agents, a new attempt to study the significance of utilisation of heterocycles hybridisation approach to furnish efficient anti-proliferative activity was reported herein. A novel series of benzofuran-isatin conjugates (**5a–e** and **7a–i**, Figure 1) linked by a carbohydrazide group, was designed and synthesised. The new compounds were screened for their potential anticancer activity following NCI, USA protocol against fifty-five different cell lines under nine different cancer panels. In addition, the cytotoxic effect of these conjugates against SW-620 and HT-29 colorectal cancer cell lines was investigated and their ability to induce cell apoptosis was examined. Furthermore, the level of the mitochondrial antiapoptotic protein Bcl2 and the level of cleaved PARP in both SW-620 and HT-29 colorectal cancer cell lines were also determined.

2. Results and discussion

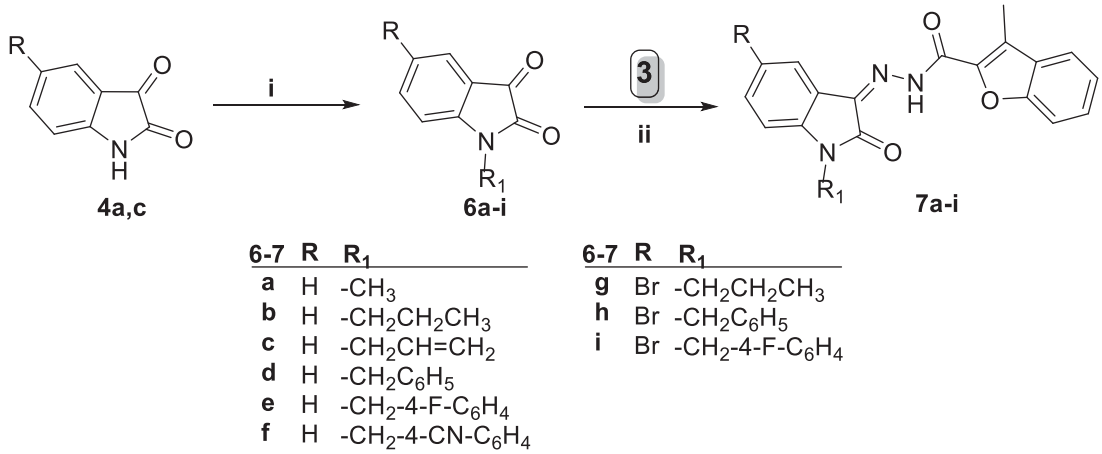
2.1. Chemistry

The adopted synthetic strategy to develop the target *N*-unsubstituted 3-methyl-*N*′-(oxoindolin-3-ylidene)benzofuran-2-carbohydrazide derivatives **5a–e** was outlined in Scheme 1.

Key starting ester 3-methylbenzofuran-2-carboxylate **2**, was prepared in 85% yield through cyclisation of 1–(2-hydroxyphenyl)ethan-1-one **1** and ethyl bromoacetate in anhydrous acetonitrile with the presence of potassium carbonate. Thereafter, heating of ester derivative **2** with hydrazine hydrate in methanol afforded the corresponding key intermediate 3-methylbenzofuran-2-



Scheme 1. Synthesis of target conjugates **5a–e**; (i) Anhydrous CH_3CN/potassium carbonate/reflux 8 h, (ii) Hydrazine hydrate/methanol/reflux 4 h, (iii) Ethanol/drops glacial acetic acid (Cat.)/reflux 3–6 h.



Scheme 2. Synthesis of target benzofurans **7a–i**; (i) (R-Br or Ar-Br)/Acetonitrile/KI (Cat.)/potassium carbonate/reflux 3 h, (ii) Ethanol absolute/drops glacial acetic acid (Cat.)/reflux 3–6 h.

carbohydrazide **3**. Finally, carbohydrazide **3** was condensed with different indoline-2,3-dione derivatives **4a–e**, through heating under reflux temperature in absolute ethyl alcohol and few drops of acetic acid, to give the desired benzofuran-based compounds **5a–e**, respectively, in 72–89%yield.

On the other hand, Scheme 2 illustrated the synthetic pathway utilised to synthesise *N*-substituted 3-methyl-*N*′-(oxoindolin-3-ylidene)benzofuran-2-carbohydrazide derivatives **7a–i**. In this scheme, alkylation of indoline-2,3-diones **4a** and **4c** was accomplished *via* heating with different alkyl bromide or benzyl bromide derivatives in anhydrous acetonitrile to produce *N*-substituted indoline-2,3-dione derivatives **6a–i**. Then indoline-2,3-diones **6a–i** were condensed with the key intermediate carbohydrazide **3** producing target benzofurans **7a–i**, respectively, in 75–87% yield.

Structures of the newly prepared benzofuran-based derivatives **5a–e** and **7a–i** were verified based on spectral and elemental analyses. ^{1}H NMR spectra of **5a–e** and **7a–i** revealed the presence of two singlet peaks for the protons of C-3 CH_3 of benzofuran ring and NH of the hydrazide linker at range δ (2.52–2.72) and (11.37–14.10) *ppm,* respectively. Moreover, structure of compounds **5a–e** was confirmed *via* presence of another singlet D_2O exchangeable signal attributable to the proton of NH for isatin moieties at δ 10.91–11.98 *ppm*. In addition, ^{1}H NMR spectra of *N*-benzyl bearing derivatives **7d–f**, **7h** and **7i** displayed the characteristic singlet signal of the benzylic protons at δ 4.98–5.07 *ppm*, while spectra for hybrids **7a**, **7b** and **7g** revealed the presence of the aliphatic protons corresponding to the *N*-substituents in these derivatives at δ (3.28 *ppm*), (0.93, 1.66 and 3.76 *ppm*) and (0.97, 1.69 and 3.80 *ppm*), respectively.

On the other hand, ^{13}C NMR spectra for the novel compounds **5a–e** and **7a–i** showed one signal belonging to the carbon of CH_3 of benzofuran ring at δ 8.12–9.49 *ppm*, also, they showed two signals belonging to the carbon of C = O functionalities for both the hydrazide linker and isatin moiety at range δ (161.15–163.62) and (164.08–167.02) *ppm*, respectively. In addition, the existence of benzylic carbon in *N*-benzyl bearing derivatives **7d–f**, **7h** and **7i** was confirmed by a signal at δ 42.14–46.03 *ppm*, whereas, the carbons of propyl moiety in compounds **7b** and **7g** appeared as signals at range δ (11.68–13.00), (20.92–22.98) and (41.30–48.74) *ppm*.

2.2. Biological evaluation

2.2.1. Nci screening of anticancer activity

In the present investigation, the chemical structures for the novel benzofuran-isatin conjugates were presented to the

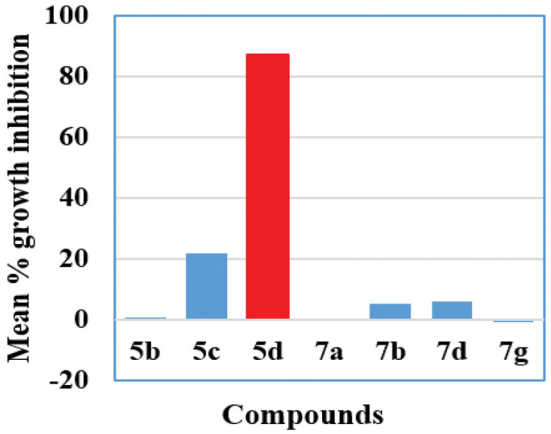


Figure 2. Mean % growth inhibition of compounds **5b–d,7a, 7b, 7d** and **7g** against NCI-55 cancer cell line panel.

Developmental Therapeutics Program at the National Cancer Institute (NCI), USA. Seven conjugates (**5b–d,7a, 7b, 7d** and **7g**) were selected, according to NCI's-DTP selection guidelines[56], for evaluating their potential *in vitro* anticancer activity against a panel of fifty-five human cancer cell lines representing nine tumour panels according to the NCI, Bethesda, Drug Evaluation Branch protocol[57,58].

2.2.1.1. Preliminary single high dose screening at 10 μM concentration. Firstly, the seven selected conjugates (**5b–d,7a, 7b, 7d** and **7g**) were screened at a dose of 10 μM for their antiproliferative activity against a panel of fifty-five cancer cell lines. The mean percent growth inhibition values (GI%) for conjugates **5b–d,7a, 7b, 7d** and **7g** against NCI-55 cancer cell lines were displayed in (Figure 2, Table 1**)**. The primary assay data analysis revealed that the new benzofuran-isatin hybrids showed weak to moderate inhibitory activity some of the subpanel cancer cell lines except for compound **5d** that possessed excellent activity against nearly all the cancer cell lines. Although compound **5b**, **7a**, **7b**, **7d** and **7g** proved inactive against most of the subpanels cancer cell lines with mean GI% = 0.75%, 0.09%, 5.18%, 6.01%, and −0.8%, respectively, they showed selective moderate anticancer activity against certain cell lines such as Ovarian-IGROV1, Non-small cell lung-EKVX, Renal-UO-31 and Breast/MCF7 cancer cell lines with GI% range 17–53% (Table 1).

In particular, compound **5d** was the most efficient anti-proliferative agent and exhibited excellent activity against almost all subpanel cancer cell lines with mean growth inhibitory activity of 87.33%. Remarkably, compound **5d** exerted excellent growth inhibition properties against Non-small cell lung cancer (NCI-H23), CNS cancer (SF-295, U251), Melanoma (LOX IMVI, SK-MEL-28), Ovarian cancer (IGROV1), Prostate cancer (DU-145) and Breast cancer (MDA-MB-468) cell lines with GI% of 89.35, 94.95, 97.95, 83.71, 84.16, 93.67, 82.38 and 80.10%, respectively (Table 1). In addition, conjugate **5d** showed good potency with GI% equals or greater than 60% towards Non-small cell lung cancer (EKVX), Colon cancer (COLO 205, HT-29 and SW-620), Melanoma (M14, MDA-MB-435 and UACC-62), Ovarian cancer (OVCAR-8), CNS cancer (SF-539), Renal cancer (SN12C), Breast cancer (MCF7, MDA-MB-231/ATCC and HS 578 T) and Prostate cancer (PC-3) cell lines with GI% of 62.73, 77.24, 72.67, 64.85, 71.32, 61.04, 65.68, 62.23, 62.58, 65.13, 77.14, 76.19, 74.50and 78.23% respectivly (Table 1).

Table 1. In vitro Anticancer screening results of compounds **5b–d,7a, 7b, 7d** and **7g** against fifty-five human tumour cell lines with single dose assay (10^{-5} M concentration). Data was provided as cell growth inhibition percentage.

Subpanel / tumour cell lines	Compound[a]						
	5b	5c	5d	7a	7b	7d	7g
Leukaemia							
CCRF-CEM	–	–	42.92	–	–	–	–
MOLT-4	–	–	49.14	–	–	–	–
HL-60(TB)	–	21.46	46.13	–	10.94	–	–
K-562	–	18.04	57.50	–	11.45	–	–
SR	–	17.62	56.52	–	–	–	–
RPMI-8226	–	–	NA	NT	NT	–	NT
Non-small cell lung cancer							
EKVX	29.63	32.13	62.73	25.07	32.35	30.23	18.10
A549/ATCC	–	27.92	123.59	–	–	–	–
HOP-92	–	26.54	143.99	–	–	12.64	–
HOP-62	–	41.41	124.09	–	18.06	20.82	–
NCI-H322M	–	31.80	69.41	–	11.87	17.83	–
NCI-H23	–	33.05	89.35	–	15.06	13.19	–
NCI-H522	10.36	10.33	45.72	–	10.32	21.72	–
NCI-H460	–	42.60	122.83	–	15.60	24.98	–
Colon cancer							
HCC-2998	–	–	56.46	–	–	–	–
COLO 205	–	–	77.24	–	–	–	–
SW-620	–	10.57	64.85	30.57	–	11.21	–
HCT-116	–	32.03	128.55	–	–	–	–
HCT-15	–	–	50.91	–	–	–	–
HT-29	–	–	72.67	–	–	–	–
KM12	–	20.46	56.20	–	–	–	–
CNS cancer							
SF-539	–	57.88	62.58	–	–	–	–
SF-268	–	11.66	42.33	–	–	–	–
SF-295	–	20.30	94.95	11.84	11.86	–	–
U251	–	42.58	97.95	–	–	–	–
SNB-19	–	31.48	57.29	–	–	11.60	–
Melanoma							
MALME-3M	12.43	64.93	129.46	–	–	–	16.04
LOX IMVI	–	33.82	83.71	–	–	13.84	–
MDA-MB-435	–	–	61.04	–	–	–	–
M14	–	25.40	71.32	–	–	–	–
UACC-257	–	–	49.98	–	–	–	–
UACC-62	10.91	30.94	65.68	–	16.86	20.56	–
SK-MEL-2	–	–	29.13	–	–	–	–
SK-MEL-28	–	–	84.16	–	–	–	–
Ovarian cancer							
NCI/ADR-RES	–	29.33	69.51	–	10.33	10.19	–
IGROV1	33.72	44.59	93.67	32.80	42.55	33.59	22.38
OVCAR-3	–	–	165.51	–	–	–	–
OVCAR-8	–	22.46	62.23	–	–	–	–
OVCAR-4	–	31.04	15,285	–	–	–	–
OVCAR-5	–	–	52.33	–	–	–	–
Renal cancer							
786-0	–	34.70	166.85	–	–	–	–
CAKI-1	19.50	26.59	151.66	19.44	11.31	18.98	14.14
ACHN	–	35.48	150.93	–	11.60	13.30	–
SN12C	–	29.81	65.13	–	–	–	–
RXF 393	–	11.08	136.15	–	–	–	–
UO-31	26.72	43.42	155.33	–	34.27	33.64	21.86
TK-10	–	–	193.95	–	–	–	–
Prostate cancer							
PC-3	–	13.75	78.23	–	10.82	15.73	–
DU-145	–	17.00	82.38	–	–	–	–
Breast cancer							
MCF7	19.04	26.66	77.14	28.25	25.04	16.66	13.20
BT-549	–	22.06	29.88	–	–	–	–
MDA-MB-231/ATCC	14.64	29.88	76.19	–	28.99	18.32	–
HS 578 T	–	46.27	74.50	–	–	–	–
MDA-MB-468	–	–	80.10	–	–	–	–
T-47D	–	42.74	139.16	21.80	23.76	33.31	–
Mean inhibition, %	0.75	21.99	87.33	0.09	5.18	6.01	−0.8
Sensitive cell lines no.	9	40	54	7	19	20	6

[a]Only GI % higher than 10% are shown. NT: not tested.

Table 2. NCI *in vitro* screening results (GI_{50}, TGI, and LC_{50} (μM) of **5d** (NSC: D-819833/1) in the five-dose test.

	Compound **5d**		
Subpanel /tumour cell lines	GI_{50}(μM)	TGI(μM)	LC_{50}(μM)
Leukaemia			
CCRF-CEM	NT	>100	>100
HL60(TB)	>100	>100	>100
K-562	NT	>100	>100
MOLT-4	NT	>100	>100
SR	NT	>100	>100
Non-small cell lung cancer			
A549/ATCC	NT	NT	NT
EKVX	2.94	>100	>100
HOP-62	1.92	4.02	8.44
HOP-92	1.84	3.98	NT
NCI-H226	2.19	NT	>100
NCI-H23	1.86	4.43	>100
NCI-H322M	NT	NT	>100
NCI-H460	NT	NT	NT
NCI-H522	6.07	>100	>100
Colon cancer			
COLO 205	NT	>100	>100
HCC-2998	NT	>100	>100
HCT-116	1.92	NT	NT
HCT-15	NT	>100	>100
HT29	NT	NT	>100
KM12	NT	>100	>100
SW-620	NT	>100	>100
CNS cancer			
SF-268	5.18	56.8	>100
SF-295	2.03	4.13	NT
SF-539	1.66	3.16	NT
SNB-19	3.45	16.2	>100
SNB-75	1.25	2.75	6.04
U251	2.03	4.26	NT
Melanoma			
LOX IMVI	3.16	>100	>100
MALME-3M	1.81	3.79	NT
M14	NT	>100	>100
MDA-MB-435	NT	>100	>100
SK-MEL-2	2.56	6.62	54.7
SK-MEL-28	NT	NT	NT
SK-MEL-5	NT	NT	>100
UACC-257	NT	>100	>100
UACC-62	5.19	>100	>100
Ovarian cancer			
IGROV1	2.10	NT	>100
OVCAR-3	1.84	NT	NT
OVCAR-4	NT	NT	NT
OVCAR-5	NT	NT	>100
OVCAR-8	3.22	>100	>100
NCI/ADR-RES	2.47	NT	>100
SK-OV-3	1.82	3.83	NT
Renal cancer			
786-0	1.99	3.84	NT
A498	1.63	4.04	NT
ACHN	1.77	NT	NT
CAKI-1	1.56	3.24	NT
RXF 393	1.79	3.70	NT
SN12C	2.82	>100	>100
TK-10	2.32	4.02	NT
UO-31	NT	NT	NT
Prostate cancer			
PC-3	NT	>100	>100
DU-145	NT	NT	>100
Breast cancer			
MCF7	NT	>100	>100
MDA-MB-231/ATCC	2.07	5.10	>100
HS 578 T	2.41	7.72	>100
BT-549	5.38	33.7	>100
T-47D	1.70	NT	>100
MDA-MB-468	2.60	6.52	>100

NT: not tested.

Table 3. Median growth inhibitory concentrations[a] (GI_{50}, μM) of *in-vitro* cancer cell lines subpanel for compound **5d**.

	Compound **5d**	
Subpanel /tumour cell lines	MG-MID	Selectivity index
non-small cell lung cancer	2.80	0.89
Colon Cancer	1.92	1.30
CNS Cancer	2.60	0.96
Melanoma	3.18	0.78
Ovarian Cancer	2.29	1.09
Renal Cancer	1.98	1.26
Breast Cancer	2.83	0.88
Full panel MG-MID[b]	2.51	

[a]Median value assessed according to the results obtained from NCI's screening.
[b]GI_{50} (μM) full panel mean-graph midpoint (MG-MID) = the average sensitivity for all cell lines towards the examined compound.

It is worthy to mention that **5d** exhibited a lethal cytotoxic impact with GI% >100 against Non-small cell lung cancer (HOP-62, A549/ATCC, HOP-92 and NCI-H460), Colon cancer (HCT-116), Melanoma (MALME-3M), Ovarian cancer (OVCAR-4 and OVCAR-3), Renal cancer (CAKI-1, 786–0, RXF 393, ACHN, TK-10 and UO-31) and Breast cancer (T-47D) cells with GI% values equals 124.09, 123.59, 143.99, 122.83, 128.55, 129.46, 152.85, 165.51, 151.66, 166.85, 136.15, 150.93, 193.95, 155.33 and 139.16%, respectively (Table 1).

On the other hand, compound **5c** showed moderate to good activity against some cell lines with mean GI% = 21.99%. The best results of compound **5c** was against cancer cell lines Non-small cell lung-HOP-62 (GI% = 41.41%), Non-small cell lung-NCI-H460 (GI% = 42.60%), Renal-UO-31 (GI% = 43.42%), Ovarian-IGROV1 (GI% = 44.59%), Breast-HS-578T (GI% = 46.27%), CNS-SF-539 (GI% = 57.88%) and Melanoma-MALME-3M (GI% = 64.93%) (Table 1).

2.2.1.2. In vitro **5 dose full NCI-55 cell panel screening..** The preliminary screening results showed that conjugate **5d** (NSC: D-819833/1) was the most potent compound in the present study, and displayed effectiveness towards various cell lines represent numerous tumour subpanels (Figure 2). Accordingly, **5d** was promoted to the five-dose (0.01–100 μM) screening assay. Accordingly, three main response parameters (GI_{50}, TGI and LC_{50}) towards each of the examined cancer cell line were calculated for hybrid **5d** and displayed in Table 2. Where, GI_{50} values represents molar concentration which produces 50% inhibitory effect in the net cell growth; TGI (cytostatic activity) is the molar concentration with total growth inhibition and LC_{50} is the cytotoxicity parameter that reflects the molar concentration that results in 50% net cell death. In addition, the mean graph midpoints (MG-MID), representing the GI_{50} average for the individual subpanels as well as the full panel cell lines were calculated giving an average potency parameter for the examined compound **5d**, (Table 3). Furthermore, by dividing the full panel MID by their individual subpanel MID, the selectivity index of compound **5d** was calculated and was used to measure the selectivity of **5d** towards different cancer cell subpanels.

Results displayed in Table 2, revealed that conjugate **5d** exhibited powerful anti-proliferative activity at a single-digit micromolar level towards all the examined human cancer cell subpanels with GI_{50} values range: 1.25 − 6.07 μM, except for Melanoma HL60(TB) cell line (more than100 μM). Moreover, regarding the cytostatic activity, hybrid **5d** exhibited excellent cytostatic activity with TGI values range 2.75–7.72 μM against numerous cell lines including NSCLC (HOP-62, NCI-H23 and HOP-92), CNS Cancer (SF-295,

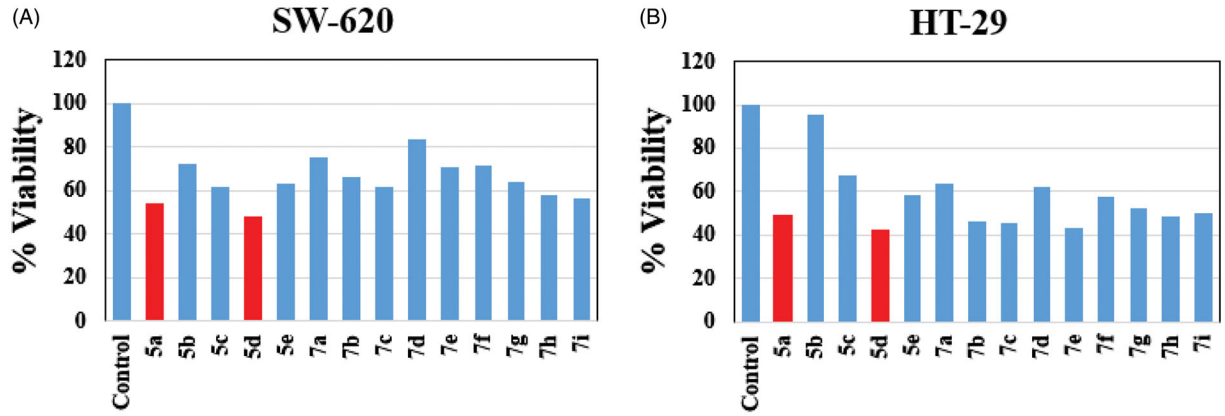


Figure 3. Effect of benzofuran–isatin conjugates (**5a–e** and **7a–i**) on the cell viability. (A) SW-620 with hybrids **5a–e** and **7a–i**, (B) HT-29 with hybrids **5a–e** and **7a–i**.

SF-539, SNB-75 and U251), Melanoma (MALME-3Mand SK-MEL-2), Ovarian Cancer (OVCAR-8), Renal Cancer (786–0, A498, CAKI-1, RXF 393 andTK-10) and Breast Cancer (MDA-MB-231/ATCC, HS 578 T and MDA-MB-468). On the other hand, while, compound **5d** showed weak to moderate cytostatic activity towards CNS Cancer (SF-268 and SNB-19), and Breast Cancer (BT-549) with TGI = 56.8, 16.2 and 33.7 μM, respectively, it proved to have no cytostatic impact (TGI >100 μM) against entire Leukaemia, Colon cancer and Prostate Cancer and the remaining examined cancer cell lines (Table 2). Furthermore, compound **5d** as revealed by the results could be considered as a non-lethal agent that exhibited LC_{50} values more than 100 μM for the all of cancer cell lines herein examined, except for three cancer cell lines; Non-Small Cell Lung Cancer (HOP-62), CNS Cancer (SNB-75) and Melanoma (MASK-MEL-2) which possessed a lethal effect of IC_{50} = 8.44, 6.04 and 54.7, respectively (Table 2).

On the other hand, as shown in Table 3, all tested subpanels were sensitive to compound **5d** with MG-MID spinning between 1.92 and 3.18 μM and the most susceptible subpanels were Colon Cancer and Renal Cancer that exhibited MG-MID = 1.92 and 1.98 μM, respectively. Furthermore, it is well known that compounds with selectivity index between 3 and 6 are considered to be of a moderate selectivity, ratios more than six indicated high selectivity towards the corresponding cell line, while compounds not meeting either of these values are considered as non-selective[59]. Therefore, as displayed in the Table 3, the calculated selectivity index for compound **5d** ranged from 0.78 to 1.30 indicated that conjugate **5d** has non-selective, broad spectrum antiproliferative activity against all tested subpanels cancer cells.

2.2.2. *In vitro* anti-cancer activity against SW-620 and HT-29colorectal cancer cell lines

In the present investigation a new set of benzofuran–isatin hybrids (**5a–e** and **7a–i**) was synthesised to be evaluated for their potential anticancer activity towards two human colorectal cancer cell lines, SW-620 and HT-29. The anticancer activity of the new conjugates was assessed using MTT assay[60], and the results were shown in Figure 3. The most active compound in the NCI assay (**5d**), in addition to another one from untested compounds by NCI (**5a**), were selected to explore their activity. Both, SW-620 and HT-29 cells were treated with 10 μM of each compound for 24 h and the percent cell viability was calculated using MTT assay. Regarding impact of the target conjugates towards SW-620 cancer cells viability, compound **5d** exhibited about 52% inhibition, whereas, compound **5a** showed 46% inhibition. On the other hand, the results showed that seven compounds (**5a**, **5d**, **7b**, **7c**, **7e**, **7h** and **7i**) showed >50% inhibition of HT-29 cancer cells viability (Figure 3).

The results revealed that compounds **5a** and **5d** exhibited promising cytotoxic activity for both cell lines. For this reason, compounds **5a** and **5d** were pursued for further studies. Starting with determination of IC_{50s} and cytotoxic selectivity studies. Serial concentrations of compounds **5a** and **5d** were used to examine their impact on cell viability using MTT protocol. Results of concentration *vs* percent viability were charted, and the IC_{50} was calculated for SW-620 and HT-29 cell lines using Graph Pad prism 8 (Figure 4). Compound **5a** was found to have IC_{50} = 9.4 μM and 8.7 μM against SW-620 and HT-29 cell lines, respectively. In addition, the IC_{50} for compound **5d** equals 9.8 μM and 6.5 μM against SW-620 and HT-29 cell lines, respectively, compared to IC_{50} of **Irinotecan**, a reference drug, which was found to be 1.0 μM against SW-620 cell line and 6.18 μM against HT-29 cell line (Figure 4).

Furthermore, selective cytotoxicity of compounds **5a** and **5d** was studied on human skin fibroblast (HFF-1) normal cells. Both conjugates were found to possess a little effect on fibroblast normal cell viability (Figure 5). These results revealed that compounds **5a** and **5d** possessed a selective cytotoxicity against SW-620 and HT-29 cancer cell lines with non-significant effect on normal fibroblast cells.

2.2.2. *Annexin V-FITC/propidium iodide apoptosis assay*

Further investigation for compounds **5a** and **5d** concerning their potential role of apoptosis induction, using Annexin V-FITC/PI double staining assay[61], was performed to evaluate their impact on both early and late apoptosis percentages in SW-620 cancer cell lines (Figure 6). The assay findings showed that compounds **5a** and **5d** resulted in a dose dependent induction of apoptosis for SW-620 cancer cells. As shown, compound **5a** induced approximately 1.7-folds and 3.8-folds total increase in apoptosis at concentration of 5 μM and 10 μM, respectively, in comparison to the control untreated SW-620 cell line (Figure 6(A)).

Similarly, compound **5d**, at concentration of 5 μM and 10 μM approximately induced 2.9-folds and 3.8-folds total increase in apoptosis, respectively, when incubated with SW-620 cell line, compared to the untreated cells (Figure 6(B)). Encouraged by these results compounds **5a** and **5d** were further investigated for their effect on the anti-apoptotic mitochondrial protein Bcl2 and their effect on the level of cleaved PARP in SW-620 colorectal cancer cell line.

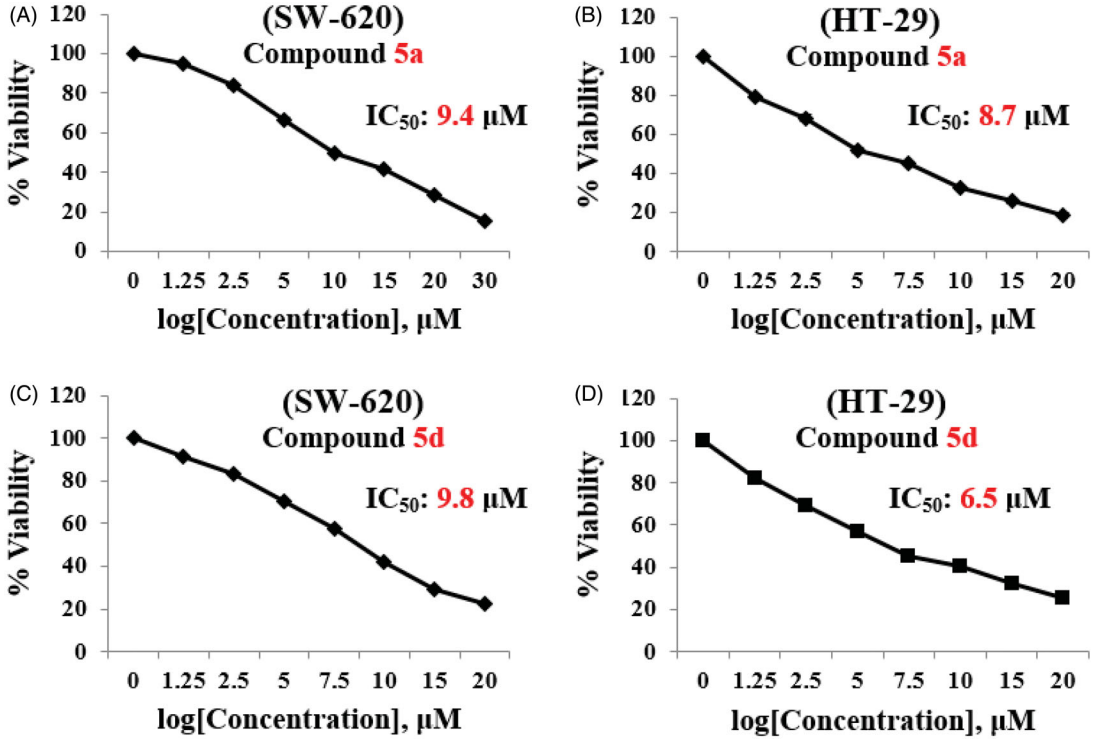


Figure 4. IC_{50} of Compound **5a** and **5d**. (A) SW-620 with compound **5a**, (B) HT-29 with **5a**, (C) SW-620 with compound **5d**, and (D) HT-29 with **5d**.

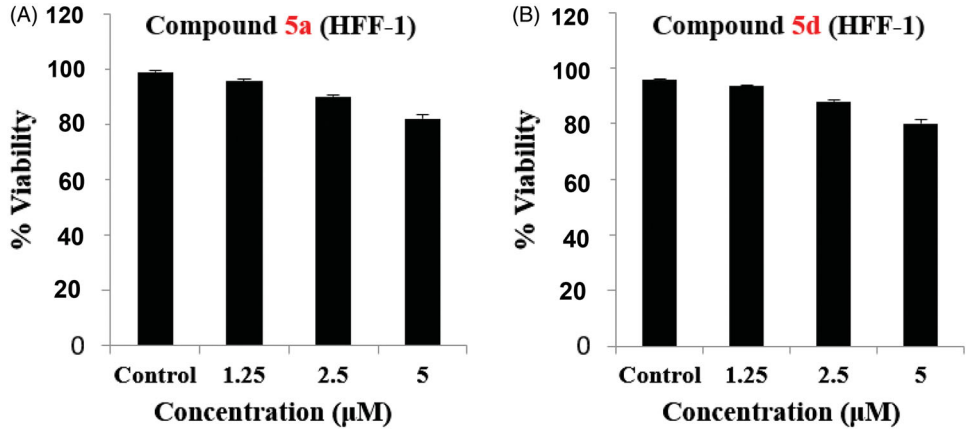


Figure 5. Impact of **5a** and **5d** on normal HFF-1 fibroblast cells, upon incubation for 24 h. (A) compound **5a** and (B) compound **5d**.

2.2.3. Effect of compounds 5a and 5d on the anti-apoptotic markers Bcl2 and the level of cleaved PARP

To further examine the possible mechanism of apoptosis, the effect of compounds **5a** and **5d** on certain apoptosis-related proteins was studied. Bcl2 protein as a critical component of the mitochondrial apoptotic pathway is reported to be overexpressed in numerous tumours causing survival of cancer cell[62]. In addition, it was reported that caspase activation during apoptosis leads to proteolytic cleavage of several cellular substrates participating in DNA reparation including [poly (ADP-ribose) polymerase][63]. Therefore, the impact of compounds **5a** and **5d** on the anti-apoptotic protein Bcl2 and the level of cleaved PARP was examined (Figure 7). The results showed that, Western blot analysis of the extracts prepared from SW-620 cells incubated with compound **5a** (5 μM and 10 μM) for 24 h, resulted in a dose dependent inhibition of Bcl2 protein expression and significant increase in the level of cleaved PARP (Figure 7(A)).

Similarly, compound **5d** was found to follow the same pattern with significant inhibition of the anti-apoptotic Bcl2 protein expression and significant increase in the level of cleaved PARP in SW-620 cancer cells (Figure 7(B)). These findings indicated that both compounds **5a** and **5d** inhibited SW-620 cells viability by deregulating apoptosis-related proteins (anti-apoptotic Bcl2 and cleaved PARP) resulting in the induction of apoptosis.

3. Conclusions

In summary, a novel series of benzofuran-isatin conjugates linked by a carbohydrazide group, (**5a–e** and **7a–i**) was designed and synthesised. Seven compounds (**5b–d** and **7a,b,d,g**) were selected according to NCI's DTP selection guidelines for the assessment of their antitumor activity against NCI-55 human cancer cell lines. All compounds proved effective against diverse cell lines among which compound **5d** was promoted to the five-dose screen and

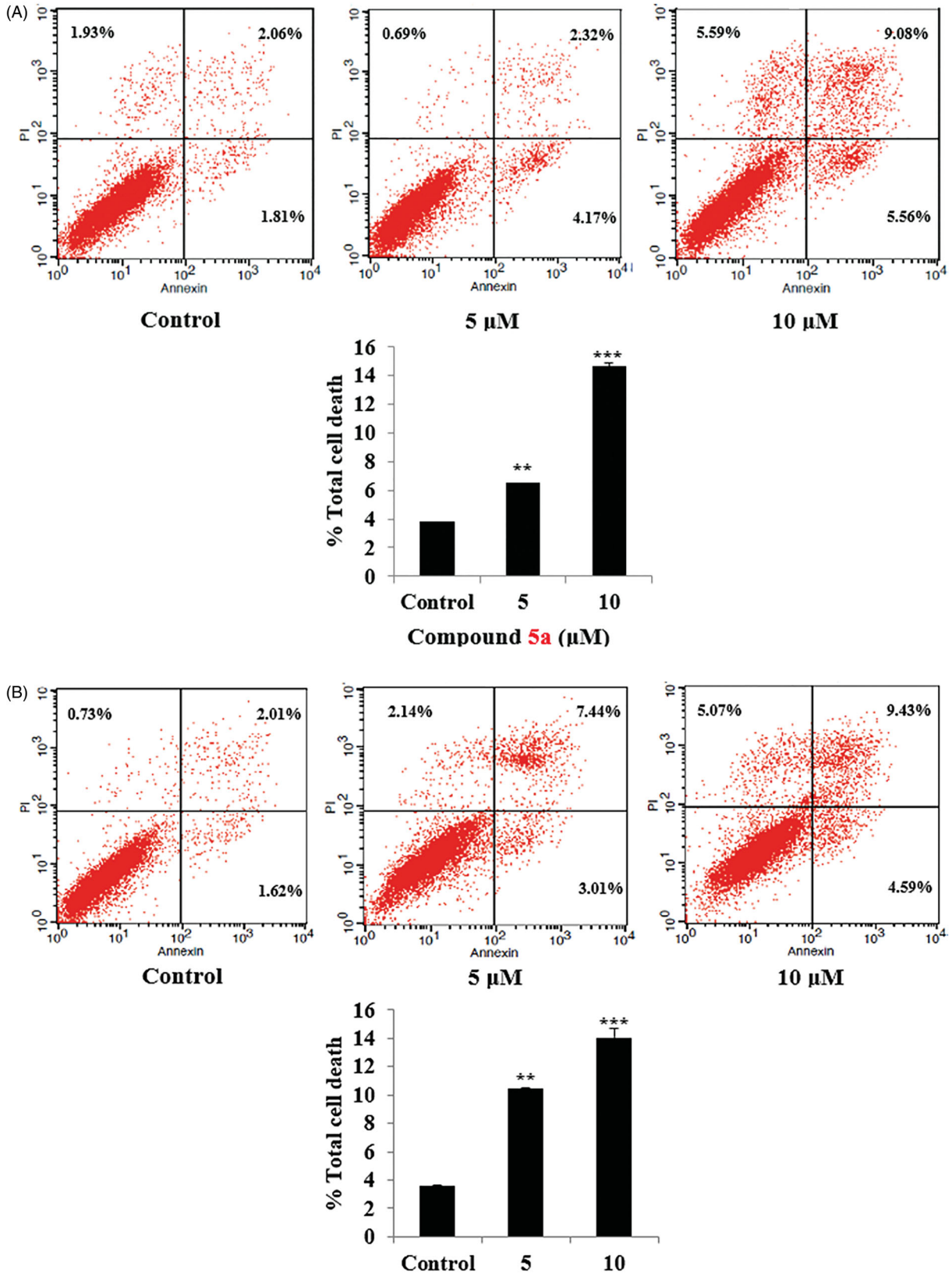


Figure 6. (A) AnnexinV/PI apoptosis assay for compound **5a**. Tow concentrations (5 and 10 µM) of compound **5a**, in addition untreated plate as a control were used to test the apoptotic effect by using Annexin V/PI in SW-620 cell line. Cells were treated with the compound **5a** for 24 h. (B) AnnexinV/PI apoptosis assay for compound 5d. Tow concentrations (5 and 10 µM) of compound **5d**, in addition untreated plate as a control were used to test the apoptotic effect by using Annexin V/PI in SW-620 cell line. Cells were treated with the compound **5d** for 24 h.

showed good to excellent growth inhibitory activity against almost all subpanel cancer cell lines. In addition, the novel conjugates (**5a–e** and **7a–i**) showed good anti-proliferative activity against two human colorectal cancer cell lines, SW-620 and HT-29, with excellent inhibitory activity for compounds **5a** and **5d** that showed IC_{50} = 8.7 µM and 9.4 µM for **5a** and IC_{50} = 6.5 µM and

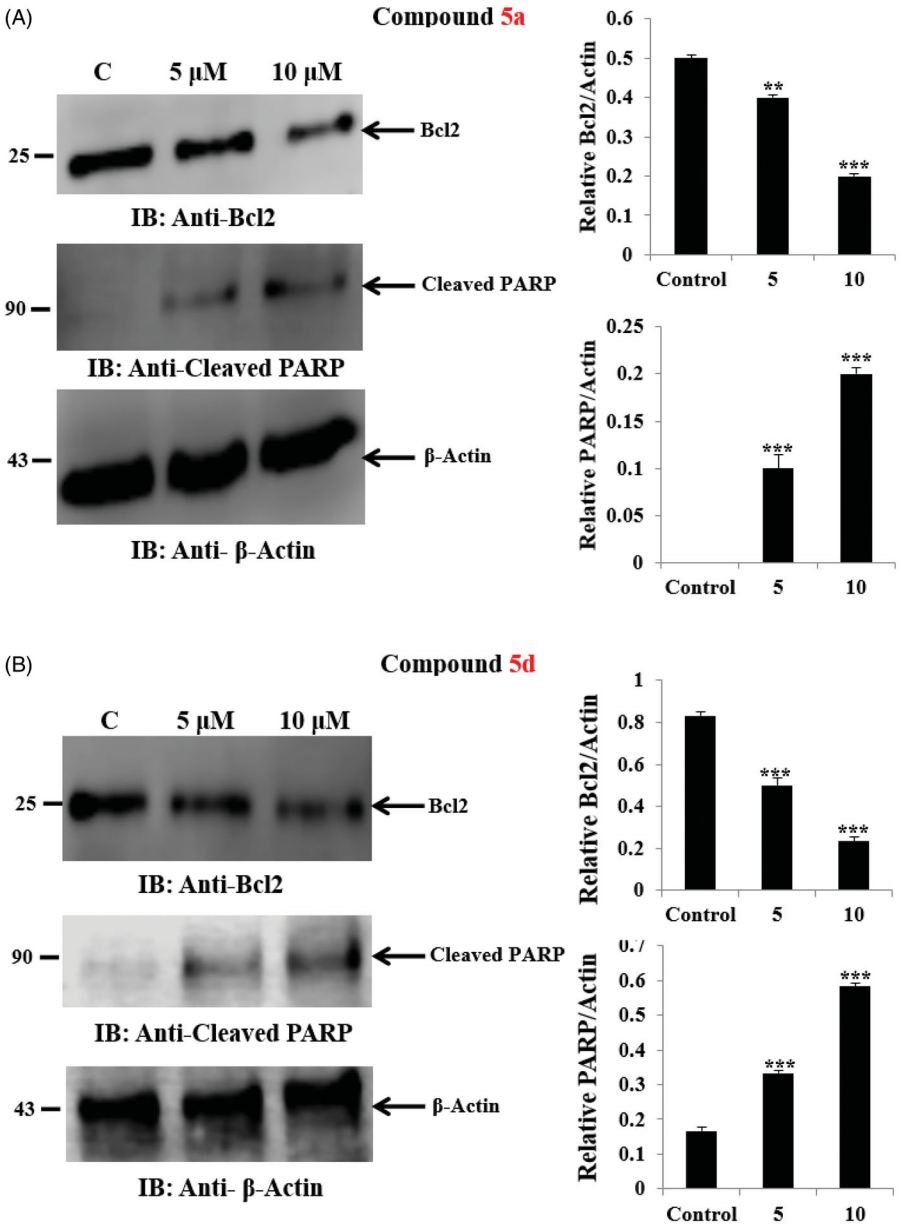


Figure 7. (A) Effect of hybrid 5a on anti-apoptotic Bcl2 protein and the level of cleaved PARP. Statistical analysis was performed where the significance of data was assessed at a p values < 0.05. *** $p < 0.001$; ** $p < 0.01$ control *vs* treated. (B) Effect of hybrid 5d on anti-apoptotic Bcl2 protein and the level of cleaved PARP. Statistical analysis was performed where the significance of data was assessed at a p values < 0.05. *** $p < 0.001$; ** $p < 0.01$ control *vs* treated.

9.8 µM for **5d** against SW-620 and HT-29 cell lines, respectively, and proved to have selective cytotoxicity with increased safety profile to fibroblast (HFF-1) normal cells. Further mechanistic studies revealed that both compounds **5a** and **5d** were able to induce apoptosis in a dose dependent manner with an approximately 1.7–3.8 folds and 2.9–3.8 folds total increase in apoptosis for compounds **5a** and **5d**, respectively, compared to the control untreated SW-620 cell line. Furthermore, both conjugates significantly inhibited the expression of the anti-apoptotic Bcl2 protein and increased the level of the cleaved PARP and resulted in SW-620 cells apoptosis. Collectively, the significant potency and high selective cytotoxicity of this series specially compounds **5a** and **5d** suggested that these conjugates might serve as starting point for additional optimisation to develop potential anticancer agents and apoptotic inducers.

4. Experimental

4.1. Chemistry

4.1.1. General

Solvents of HPLC grade have been used and purchased from Thermo Fisher. Follow up of reactions has been performed utilising precoated TLC F_{254} Merck plates. Schimadzu FT-IR spectrometer has been used for functional groups analysis for the synthesised derivatives. NMR spectrometric analyses have been conducted using Bruker-Avance 400 NMR spectrometer (100 MHz for ^{13}CNMR and 400 MHz for ^{1}H NMR). Chemical shifts have been recorded in *ppm*. Multiplicities have been reported with their 1st order J coupling constants (Hz) for doublets (d); Stuart apparatus has been used to determine the melting points. FLASH 2000 CHNS/O analyser has been adopted to perform the elemental

analysis. Compounds **3**[64], and **6a–i**[65,66] have been reported previously.

4.1.2. Synthesis of target derivatives 5a–e and 7a–i

To stirred hot solution of 3-methylbenzofuran-2-carbohydrazide **3** (0.25 g, 1.3 mmol) in 13 ml of absolute EtOH with catalytic drops of ethanoic acid, equivalent amount of appropriate indoline-2,3-dione compounds **4a–e** or **6a–i** has been added. The reaction mixture has been then refluxed for (3–6) h. The produced precipitate, after cooling, was collected by filtration, washed with water then recrystallized from glacial acetic acid to produce target derivatives **5a–e** and **7a–i**, respectively in a good yield (70–87%).

Full characterisation (NMR, IR, and elemental analysis) data for target compounds (**5a–e** and **7a–i**) have been presented in the Supporting Materials.

4.2. Biological evaluation

All *in vitro* biological assays in this study; NCI anticancer screening[67,68], MTT cell viability assay[54], Annexin V-FITC/PI assay[54] and Western blot analysis[54] were performed as reported earlier. All experimental procedures were provided in the Supporting materials.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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