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Synthesis, Cytotoxic, and Apoptotic Activity of New *N*-(Arylglycyl)succinohydrazide and their Sugar Hydrazone Derivatives

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Abstract:

Hydrazones have pronounced properties that allow them promising for a range of synthetic applications. In the current study, new acid hydrazide compounds incorporating substituted arylglyl and succinyl moieties were prepared *via* multistep process including formation of the corresponding acid compounds followed by formation of the derived esters and hydrazinloysis reactions. The synthesized acylhydraizdes were used for the preparation of novel functionalized sugar hydrazones and their derived per-*O*-acetylated derivatives. The hydrazones and their acetylated analogs were studied for their cytotoxic and apoptotic activity against colorectal HCT116, hepatic HepG2, and breast MCF7 cancer cell lines, and it was revealed that hydrazones **16**, **17,** and **18** possessing per-*O*-acetylated sugar moieties showed the highest cytotoxic activity and Bax/Bcl-2 genetic ratio compared to the other compounds and the reference drug**.**

Keywords: arylglycyl, hydrazides, sugar hydrazone, cytotoxicity, anticancer.

Introduction

Cancer diseases represent an increasing threat to human health because of their terrifying effects due to the increase in the number of deaths due to such threat, which represents an urgent motive to intensify research in the field of medicinal chemistry. Chemotherapy is involved in important applied treatment strategies and consequently, the need for potent bioactive functionalized compounds of various characteristics with less or minimal toxicities is an important objective [1].

Interestingly, hydrazide-hydrazone compounds have been revealed, *via* emphatic studies, through its remarkable diversity of properties and the widespread interest in organic and medicinal chemistry [2–7]. Incorporation of the azomethine functionality linked to carbonyl as in the >C = N-NH-CO linkage, Hydrazones are allowed as a versatile group of organic compounds of great importance, which are widely applied as ligands in coordination chemistry. [8,9]. A survey of the literature showed that the hydrazide and hydrazone fractions play an important role in the anticancer activity of the nitrogen - containing heterocycles [10]. Furthermore, hydrazones and their complexes have gained significant interest due to the reported broad spectrum of their biological activities including anti-carcinogenic [11-18], anti-inflammatory [19], anticonvulsant [20], analgesic [21], antimicrobial [22-25], anti-inflammatory Fungi [26], and anti-tuberculosis activities [27,28]. Aroylhydrazones, as certain derivatives of particular interest that show particularly effective and selective anti-proliferative activities [29-31], as well as potential inhibition of DNA synthesis and cell growth in various cultured human and rodent cells [32].

On the other hand, organic compounds incorporating sugar moieties as structurally modified analogs of nucleosides found considerable interest since their synthesis and bioactivity investigation as inhibitors, anticancer and antiviral candidates were extensively studied [33-38]. Sugar hydrazones are considered as analogs of modified glycoside and acyclic nucleosides and functionalized derivatives are expected to be of biological and synthetic interest. The above significances and our group interest in the synthesis and investigation of anticancer activities of newly synthesized compounds incorporating sugar moieties promoted us, in the current study, for the

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synthesis of new functionalized sugar hydrazones linked to succinyl and arylglycyl moieties.

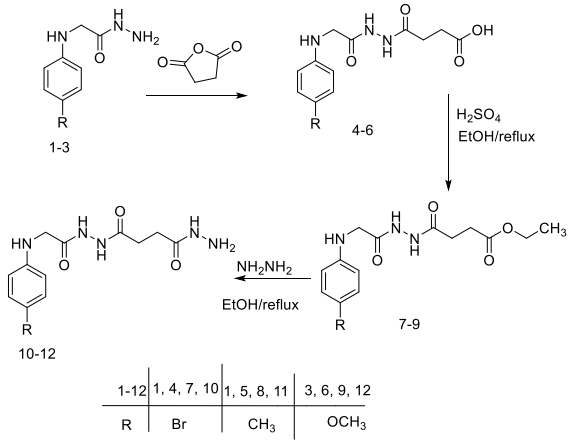
RESULTS AND DISCUSSION

CHEMISTRY

The synthetic strategy depended mainly on the possibility of ring opening reaction of succinic anhydride by means of acid hydrazide which affords poly-functionalized acid. A series of *N*-arylglycinyl hydrazides **1-3** [39,40] were used as starting substrates which were allowed to react with succinic anhydride to yield their corresponding succino-hydrazide derivatives **4-6** in moderate yields. Infrared spectra of the resulting 4-6 carboxylic acid compounds showed the absorption bands of the hydroxyl groups in the carboxylic functions of their distinct regions as well as the remaining functions such as the carbonyl and NH groups. Their H NMR spectra indicated the presence of four methylene protons in addition to the signals assigned to OH and aromatic protons.

Esterification of the succinoglycinyl acid derivatives via treatment with absolute ethanol in acidic medium resulted in the formation of their respective corresponding ethyl ester products 7-9 in moderate yields. Their infrared spectroscopic data revealed the absorption at their characteristic band values as well as the disappearance of the carboxyl frequency bands of the starting acid compounds. In addition, their 1H NMR spectra showed the characteristic signals of the ethyl group as triplet and quaternary of CH_3 and CH_2, respectively, as well as signals of the residual methylene protons and aryl proton signals confirming the assigned structures. Hydrazinolysis of subsequent ester derivatives through their interaction with hydrazine hydrate in ethanol leads to the formation of acylhydrazide derivatives 10-12 (Scheme 1). The structures of the subsequent hydrazide products were confirmed by their spectroscopic data such as infrared and NMR spectra that revealed confirmation of the assigned structures. Thus, their IR spectra showed the carbonyl amide absorption bands at 1680-1690 cm−1 as well as the NH and NH_2 bands in their distinct regions. 1H NMR spectra showed amino and NH signals as well as residual signals for the the assigned structure in addition the disappearance of ethyl signals in the corresponding ester substrates. (**Scheme1**).

The synthesized acid hydrazides were used for the preparation of a number of sugar hydrazones via their reaction with an aldehyde sugar. Thus, reacting the succino-hydrazide derivatives **10-12** with D-xylose in an acidic medium produced the corresponding sugar hydrazone compounds **13-15** in good yields. Infrared spectra of succinohydrazone bound to sugar derivatives showed the absorption attributed to the hydroxyl groups of the sugar fraction in their distinct bands. The NMR spectra of the resulting sugar hydrazones showed H-1 from the sugar fraction at 7.52-7.55 ppm indicating the association of the sugar fraction in its non-cyclic form due to the high chemical shift value observed as a result of the sp^2 nature of the C-1 carbon. It has been reported that the H-1 chemical shift of hydrazinyl sugar derivatives to possess cyclic sugar moieties appears at lower chemical shift values (5.80-6.00 ppm) [41,42]. The NMR spectra also showed the signals of sugar hydroxyl and residual hydrogen in the sugar chains as well as aryl and methylene protons.



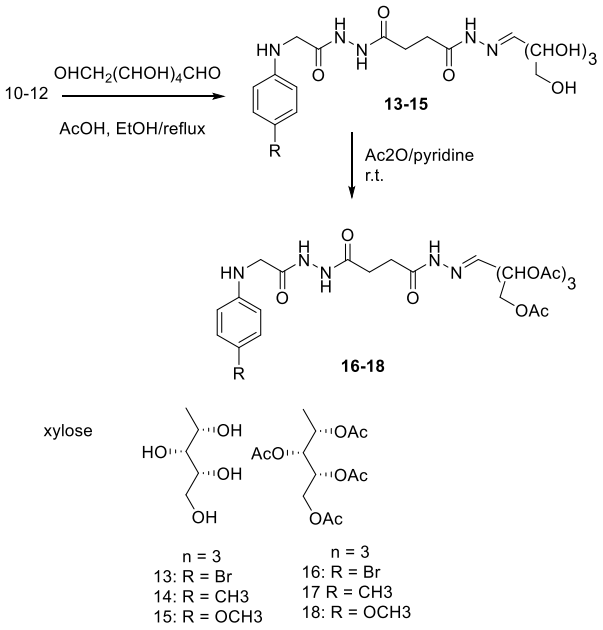
Scheme 1: Synthesis of N-arylglycenyl hydrazinyl derivatives

The synthesized sugar hydrazones were converted to the derived acetyl hydrazones by reaction with acetic anhydride in pyridine at room temperature resulted in the formation of per-O-acetylated acetylated derivatives 16-18 (Scheme 2). In the infrared spectra of the latter, acetyl hydrazones revealed the presence of carbonyl group bands characteristic of the $OCOCH_3$ groups and also the disappearance of the hydroxyl absorption bands from the primary sugar hydrazones. In the 1H NMR spectra of per-O-acetylated compounds 16-18, the assigned signals attributed to acetylmethyl protons at their characteristic values confirmed that in addition to the protons of the sugar moiety, the CH_2 groups and aryl protons also confirmed the structures (**Scheme 2**).

ANTICANCER SCREENING

In the present study, the newly synthesized compounds examined their anticancer activities *in vitro* by testing their cytotoxic activities against human colorectal HCT116, hepatic HepG2, and breast MCF7 cancer cell lines. The present results showed that there was a significant ($P < 0.05$) gradual decrease in cell proliferation after treatment of the three introduced cancer cell lines with the synthesized compounds utilize different dosages (0, 20, 40, 60, 80, 100 µM), as shown in (Figs. 1-3). Zero concentration represents the untreated cancerous cells with the synthesized compounds and used as a

negative control. 5-Flurouracil (5-FU), standard anti-cancer drug, was used as a positive control.



Scheme 2: Synthesis of sugar hydrazone derivatives cntaining arylglaycinyl moiety

Table **1** shows the values of the half-maximal inhibitory concentrations (IC_{50}), which are the concentrations that kill 50% of the cancer cells. It can be said from (Figs 1-3) and (Table 1) that the lower the IC_{50}, the greater the cytotoxic effect against HCT116, HepG2 and MCF-7 cancer cell lines. Compounds **16, 17,** and **18** were recorded as the lowest IC_{50s} with the highest cytotoxic effects against all cancer cell lines as shown in (Figs. 1-3) and (Table 1), as compared to other synthesized compounds.

For human breast MCF7 cell line, Compounds **16, 17,** and **18** were recorded as the lowest IC_{50s} (25.81, 29.96 and 37.37 µM, respectively) versus the positive control (5-FU; IC_{50} = 64.00 µM), with the highest cytotoxic effects as shown in (Figure 3) and (Table 1). Thus, we can suggest that the breast cancer cell line MCF7 is the most sensitive cell type to compounds **16, 17**, and **18** followed by the hepatic cancerous HepG2 cells (i.e. promising IC_{50} values but higher than that of MCF7 cell line: 36.88, 36.90 and 45.60 µM, respectively) versus the positive control (5-FU; IC_{50}= 76.46 µM), with high cytotoxic effects as illustrated in (Fig 2 and Table 1).

Eventually, Compounds **16**, **17**, and **18** exhibited the lowest cytotoxic effects against human colorectal HCT 116 cells, with higher IC_{50S} (41.33 µM, 41.91 µM, 53.80 µM, respectively) than human HepG2 cells and human breast cancer MCF7 cells, as indicated in (Fig. 1 and Table 1).

Furthermore, the recent activity for compounds **16**, **17** and **18** against human colorectal HCT116 cells was compared with the positive control (5-FU; IC_{50}= 90.08 µM), with high cytotoxic effects as illustrated in (Fig 1 and Table 1).

Our study indicated that the promising anti-cancer activities of compounds **17** and **18** against breast MCF7, then liver HepG2, then colorectal HCT116 cancerous cells. This reflected that our newly synthesized compounds were most potent and can be used as a therapeutic chemoprevention against these cancerous cell lines compared to 5-Flurouracil, the currently available anti-cancer drug against solid tumors.

In an attempt for correlation of the afforded activity results against the cancer cell lines in the current investigation, the importance of the attachment of sugar moiety to the hydrazide compounds *via* hydrazone linkage was obviously cleared. The latter observation was revealed as the activity was raised for the synthesized acetylated suga hydrazones. On the other hand, the sugar hydrazones **16-18** incorporating the acetylated sugar moieties showed higher activities than their free-hydroxy precursors **13-15**.

Table (1): IC_{50s} of compounds against colorectal, liver and breast cancer cells.

Cpds/ IC_{50} (µM)	HCT 116 cells	HepG2 cells	MCF-7 cells
13	58.13	50.12	38.56
14	55.07	47.27	37.67
15	62.38	53.51	42.92
16	41.33	36.88	25.81
17	41.91	36.90	29.96
18	53.80	45.60	37.37
5-FU	90.08	76.46	64.00

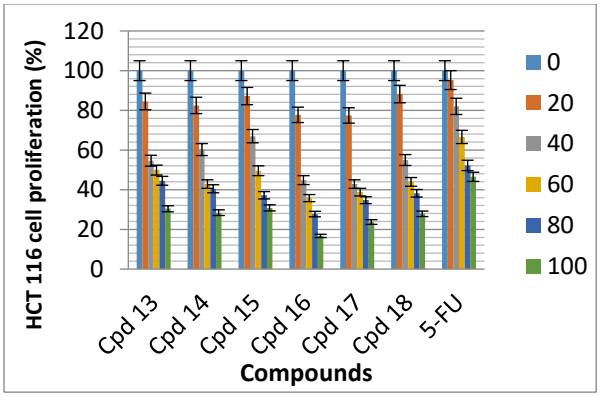


Figure 1. Anti-proliferative activities against HCT 116 cancer cells.

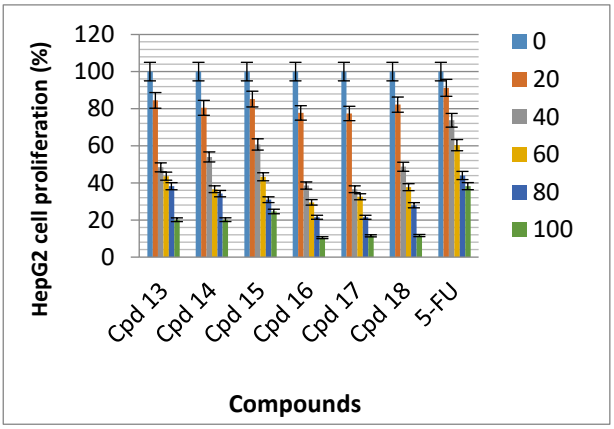


Figure 2. Antiproliferative activities of compounds against human hepatocellular carcinoma HepG2.

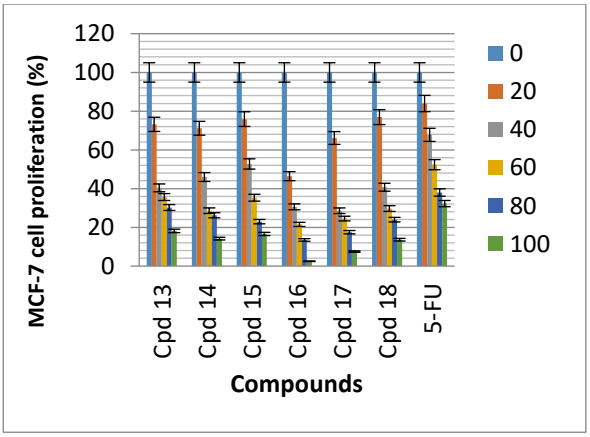


Figure 3. Anti-proliferative activities of the compounds against MCF-7 human breast cancer cells.

Furthermore, the acetylated compounds possessing the *para*-methyl- and *para*-bromoarylaminoglycyl system showed relatively higher activities than their analogue with the methoxy substituent. Such observation was also revealed regarding the sugar hydrazones with free un-acetylated hydroxyl groups.

Genetic expression of Bax and Bcl-2 genes

To explore the mechanistic way of cancer cell death, we tested the gene expressions of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) genes in colorectal HCT116, hepatic HepG2 and breast MCF7 cancerous cell lines, Compound **16** acquired the highest significant up-regulation of Bax gene expression in MCF-7, then HepG2 followed by HCT116 cells compared to the control. The acetylated sugar hydrazone **16** was superior in its induction of Bax genetic expression compared to its analogues **17** and **18** (Fig. 4).

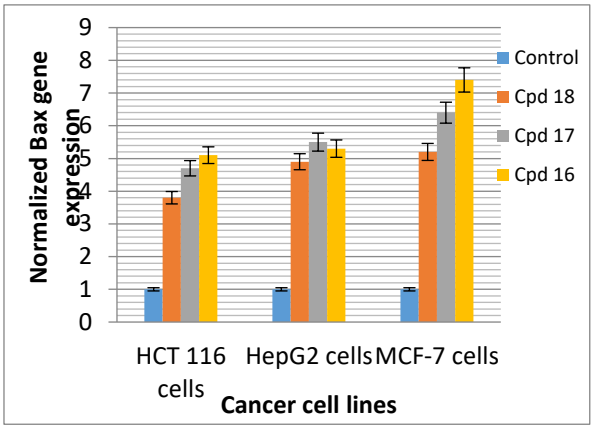


Figure 4. Effect of compounds 16-18 against the pro-apoptotic gene (Bax gene).

Compound **16** made the highest down-regulation of Bcl-2 gene expression in MCF-7, then HepG2 and HCT116 cells, respectively compared to the control. Compound **16** was superior in its inhibition of Bcl-2 genetic expression compared to Compounds **17** and **18** (Fig. 5).

The ratio of proapoptotic (Bax) and anti-apoptotic (Bcl-2) gene expressions is very important to confirm the apoptotic effect of any new treatment, so we calculated this ratio and proved that compound 16 recorded the highest Bax/Bcl-2 ratios In MCF-7 cells (83.14) and HepG2 cells (53.00) by the control, followed by methyl substituted analog 17 which recorded high Bax/Bcl-2 ratios in MCF-7 cells (21.33) and HepG2 cells (9.16) normalized by the control. On the other hand, derivative **18** showed Bax/Bcl-2 ratios in MCF-7 cells (13.00) and HepG2 cells (8.16) normalized by the control (Table 2).

One of the possible modes of action and behavior of tested compounds, according to reported anticancer studies of similar synthesized structures incorporating hydrazinyl sugar moieties, is their possible enzyme inhibition activity of kinases such as CDK2 enzyme and EGFR [43,44] which are known to be involved in the process.

On correlation of the revealed results with the structural features of the tested compounds, the effect of acetylation appeared to result in more active compounds as the resulting acetylated compounds showed higher activities than their free hydroxyl precursors. In addition, the MCF-7 cells were the most susceptible by the para-substituted bromo-aryl and para-methyl-aryl substituent which showed higher activities than the methoxy isostatic analog.

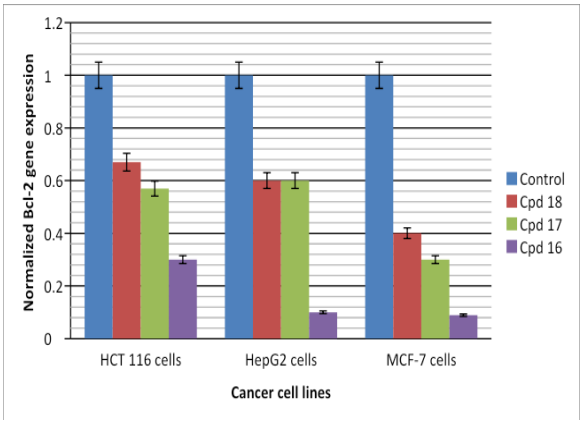


Figure 5. Effect of the compounds **16-18** against the anti-apoptotic gene (Bcl-2 gene).

Table 2: The Ratio of proapoptotic (Bax) and anti-apoptotic (Bcl-2) gene expressions.

Bax/Bcl-2 ratio	Cpd 16	Cpd 17	Cpd 18
HCT 116 cells	17.00	8.24	5.67
HepG2 cells	53.00	9.16	8.16
MCF-7 cells	83.14	21.33	13.00

EXPERIMENTAL

CHEMISTRY

Materials and scientific methods:

All organic compounds and solvents have been purchased from Fluka, Acros or Merck, Naser City, Egypt. All used chemicals were used in their analytical grade (BDH, Sigma or Aldrich) and without more purification. Infrared spectra performed on a Nicolet FT-IR spectrophotometer in the range 4000-400 cm^{-1}. The ^1H NMR spectra recorded in DMSO-d_6 on a Varian Gemini 200 NMR spectrometer at 300 MHz. Fast Atom Bombartment (FAB) mass spectra for the ligands carried out on a Shimadzu Qp-2010 Plus spectrometer. Melting points have been measured by utilizing Stuart melting point apparatus. Elemental analysis (C, H, and N) were performed on a Perkin Elmer-2400 elemental analyzer at Main Defence Chemical Laboratory.

General procedures for the preparation of 2-((4-Substituted-aryl)amino)acetohydrazides (1-3)

2-((4-Bromophenyl)amino)acetohydrazide was prepared as mentioned [39,40]. An excess of hydrazine hydrate (5 mL) was added to an ester suspension (3 g, 0.011 mmol) in ethanol (40 mL). The reaction mixture was refluxed for 6 hours and then poured into ice cold water with stirring, the solid product was collected by filtration, dried, and recrystallized from ethanol to give the pale yellow hydrazide crystals (2.7 g, 90 %).

General procedures for the preparation of acid hydrazides(4-6).

A mixture of hydrazides **1-3** (3 g, 0.012 mmol) and succinic anhydride (2.5 g, 0.025 mmol) was stirred into toluene (30 ml) at room temperature for 24 h. The solvent was largely removed under reduced pressure and then cooled down and the remaining solid product was filtered, dried, and crystallized from ethanol to give the acidic derivatives.

4-(2-(2-((4-Bromophenyl)amino)acetyl)hydrazinyl) -4-oxobutanoic acid (4).

Yield: 80%; m.p. 179-180 °C; IR (KBr) cm^{-1}, 3205-3190 (OH), 3384 (NH), 3205 (NH), 3045 (CH-arom.), 2923 (CH-aliph), 1693 (C=O), 1632 (C=O), 1491 (C=C). ^1H NMR (DMSO-d_6) ppm, δ: 2.39 (2H, t, J = 6.2 Hz, CH_2), 2.47 (2H, t, J = 6.2 Hz, CH_2), 3.07 (2H, s, CH_2), 7.18 (1H, d, J = 8.5 Hz, Ar-H), 7.42 (1H, d, J = 8.5 Hz, Ar-H), 7.51 (1H, t, J = 5.8 Hz, NH), 9.03 (1H, s, CONH), 9.26 (1H, s, CONH), 10.01 (1H, s, COOH). ^{13}C NMR (DMSO-d_6) ppm, δ 39.2, 56.2, 62.5 (3 CH_2), 129.8 -133.6 (Ar-C), 169.4, 169.9, 170.5 (3 C=O). Analysis calcd. for $C_{12}H_{14}BrN_3O_4$: C, 41.88; H, 4.10; N, 12.21. Found: C, 41.59; H, 4.14; N, 12.07.

4-Oxo-4-(2-(2-(p-Tolylamino)acetyl)hydrazinyl) butanoic acid (5).

Yield: 79%; m.p. 177-178 °C; IR (KBr) cm^{-1}, 3200-3170 (OH), 3342 (NH), 3282 (NH), 3048 (CH-arom.), 2962 (CH-aliph), 1741 (C=O), 1683 (C=O), 1521 (C=C). ^1H NMR (DMSO-d_6) ppm, δ: 2.22 (3H, s, CH_3), 2.30 (2H, t, J = 6.2 Hz, CH_2), 2.40 (2H, t, J = 6.2 Hz, CH_2), 3.60 (2H, s, CH_2), 5.77 (1H, s, NH), 6.47 (1H, d, J = 8.5 Hz, Ar-H), 6.88 (1H, d, J = 8.5 Hz, Ar-H), 9.75 (1H, s, CONH), 9.81 (1H, s, CONH), 10.11 (1H, s, COOH). ^{13}C NMR (DMSO-d_6) ppm, δ 20.1 (CH_3), 37.4, 50.2, 60.1 (3 CH_2), 129.5 -145.6 (Ar-C), 169.9, 170.2, 170.4 (3 C=O). Analysis calcd. for $C_{13}H_{17}N_3O_4$: C, 55.91; H, 6.14; N, 15.05. Found: C, 55.71; H, 6.17; N, 14.90.

4-(2-(2-((4-Methoxyphenyl)amino)acetyl) hydrazinyl)-4-oxobutanoic acid (6).

Yield: 75%; m.p. 169-170 °C; IR (KBr) cm^{-1}, 3208-3185 (OH), 3336 (NH), 3282 (NH), 3110 (CH-arom), 2925 (CH-aliph), 1741 (C=O), 1681 (C=O), 1513 (C=C). ^1H NMR (DMSO-d_6) ppm, δ: 2.19 (2H, t, J = 6.2, CH_2), 2.26 (2H, t, J = 6.2, CH_2), 3.61 (m, 2H, CH_2), 3.63 (3H, m, CH_3), 5.88 (1H, br, NH), 6.69 (1H, d, J = 8.5 Hz, Ar-H), 6.92 (1H, d, J = 8.5 Hz, Ar-H), 7.29 (1H, s, CONH), 7.46 (1H, s, CONH), 9.82 (1H, s, COOH). Analysis calcd. for $C_{13}H_{17}N_3O_5$: C, 52.88; H, 5.80; N, 14.23. Found: C, 52.69; H, 5.72; N, 14.41.

General procedures for the preparation of ester (7-9)

A mixture of the substituted arylamino-succenic acid derivatives **4-6** (0.058 mmol), absolute ethanol (25 mL) and conc. H_2SO_4, (1 mL) was heated under reflux for 6-8 hours as judged by TLC to indicate completion of the reaction. The excess ethanol was largely removed under reduced pressure and then cooling to room temperature with standing for 5 hours gave the ester derivatives **7-9**, respectively.

Ethyl-4-(2-(2-((4-bromophenyl)amino)acetyl) hydrazinyl)-4-oxobutanoate (7).

Yield: 73%; White foam; IR (KBr) cm^{-1}: 3421 (NH), 3139, (CH arom), 2985 (CH aliph), 1740 (C=O), 1621 (C=O), 1511 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 1.11 (3H, t, *J* = 5.8 Hz, CH_3), 2.24 (2H, t, *J* = 6.2 Hz, CH_2), 2.46 (2H, s, CH_2), 3.66 (2H, s, CH_2), 3.77 (2H, q, *J* = 5.8 Hz, CH_2), 5.90 (1H, br, NH), 6.57 (1H, d, *J* = 8.5 Hz, Ar-H), 7.1 (1H, d, *J* = 8.5 Hz, Ar-H), 7.33 (1H, s, CONH), 7.52 (1H, s, CONH). ^{13}C NMR (DMSO-d_6) ppm, *δ* 17.2 (CH_3), 33.5, 39.5, 61.5, 62.1 (4 CH_2), 127.8 -140.6 (Ar-C), 169.2, 169.7, 170.6, 170.9 (4 C=O). Analysis calcd. for $C_{14}H_{18}BrN_3O_4$: C, 45.18; H, 4.87; N, 11.29. Found: C, 45.41; H, 4.72; N, 11.22.

Ethyl-4-oxo-4-(2-(2-(p-tolylamino)acetyl) hydrazinyl)butanoate (8).

Yield: 73%; White foam; IR (KBr) cm^{-1}: 3658 (NH), 3117 (CH arom), 2985 (CH aliph), 1719 (C=O), 1620 (C=O), 1510 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 1.65 (3H, t, *J* = 6.4 Hz, CH_3), 2.20 (2H, t, *J* = 6.8 Hz, CH_2), 2.27-2.36 (5H, m, CH_2, CH_3), 4.07-4.15 (4H, m, 2CH_2), 6.02 (1H, d, *J* = 8.5 Hz, Ar-H), 6.01 (1H, br, NH), 6.04 (1H, d, *J* = 8.5 Hz, Ar-H), 7.11 (1H, s, CONH), 7. 14 (1H, s, CONH). Analysis calcd. for $C_{15}H_{21}N_3O_4$: C, 58.62; H, 6.89; N, 13.67. Found: C, 58.42; H, 6.81; N, 13.75.

Ethyl-4-(2-(2-((4-methoxyphenyl)amino)acetyl) hydrazinyl)-4-oxobutanoate (9).

Yield: 70%; White foam; IR (KBr) cm^{-1}: 3423 (NH), 3095 (NH), 3004 (CH arom), 2925 (CH aliph), 1733 (C=O), 1657 (C=O), 1511 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 1.60 (3H, t, *J* = 5.8 Hz, CH_3), 2.25 (2H, t, *J* = 6.2 Hz, CH_2), 2.29 (2H, t, *J* = 6.2 Hz, CH_2), 3.65 (3H, s, OCH_3), 3.69 (2H, q, *J* = 5.8 Hz, CH_2), 5.03 (1H, t, *J* = 5.8 Hz, NH), 7.11 (1H, d, *J* = 8.5 Hz, Ar-H), 7.23 (1H, d, *J* = 8.5 Hz, Ar-H), 7.27 (1H, s, CONH), 7.40 (1H, s, CONH). Analysis calcd. for $C_{15}H_{21}N_3O_5$: C, 55.72; H, 6.55; N, 13.00. Found: C, 55.92; H, 6.59; N, 12.88.

***N'*-(2-((4-Substituted-aryl)amino)acetyl) succinohydrazide (10-12)**.

Hydrazine hydrate (2 mL) were added to a solution of ethyl ester derivatives **7-9** (0.033 mmol) in ethanol (25 mL). The reaction mixture heated under reflux for 8 hours with stirring. After cooling to room temperature, collected the solid product by filtration, then dried, and recrystallized from ethanol to give the corresponding hydrazide derivatives **10-12**, respectively.

***N*'-(2-((4-Bromophenyl)amino)acetyl) succinohydrazide (10).**

Yield: 72%; m.p. 157-158 °C; IR (KBr) cm^{-1}: 3374, 3325 (NH_2, NH), 3050, (CH arom), 2982 (CH aliph), 1684 (C=O), 1594 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 2.50-2.55 (m, 4H, CH_2), 3.44 (2H, s, CH_2), 4.23 (2H, br, NH_2), 6.08 (1H, t, *J* = 5.8 Hz, NH), 6.49 (1H, d, *J* = 8.5 Hz, Ar-H), 7.18 (1H, d, *J* = 8.5 Hz, Ar-H), 9.12 (2H, br, 2NH), 9.14 (br, 1H, NH). EI-MS: m/z: 360.67 [M^+]. Analysis calcd. For $C_{12}H_{16}BrN_5O_3$: C, 40.24; H, 4.50; N, 19.55. Found: C, 40.17; H, 4.61; N, 19.68.

N'-(2-(*p*-Tolylamino)acetyl)succinohydrazide (11).

Yield: 77%; m.p. 154-155 °C; IR (KBr) cm^{-1}: 3352, 3302 (NH_2, NH), 3199 (NH), 3019 (CH arom), $_{2917}$-2895 (CH-aliph), 1657 (C=O), 1521 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 2.05-2.18 (4H, m, 2CH_2), 2.25 (3H, s, CH_3), 3.71-3.90 (4H, m, NH_2, CH_2), 6.42 (1H, s, NH), 6.84 (2H, d, *J* = 8.5 Hz, Ar-H), 7.30 (2H, m, Ar-H), 7.40-7.48 (2H, br, 2NH), 8.94 (1H, s, NH). EI-MS: m/z ($C_{13}H_{19}N_5O_3$): 309 [M^+]. Analysis calcd. for C13H19N5O3: C, 53.23; H, 6.53; N, 23.88. Found: C, 53.05; H, 6.58; N, 23.97.

***N*'-(2-((4-Methoxyphenyl)amino)acetyl)succinohydrazide (12).**

Yield: 77%; m.p. 159-160 °C; IR (KBr) cm^{-1}: 3349-3304 (NH_2, NH), 3288 (NH), 3199 (CH-arom), 2949-2927 (CH aliph), 1655 (C=O), 1517 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 2.35-2.47 (4H, m, 2CH_2), 3.61-3.65 (5H, m, CH_2, OCH_3), 5.01 (2H, br, $_{NH2}$), 5.80 (br, 1H, NH), 7.26 (2H, d, *J* = 8.5 Hz, Ar-H), 7.29 (2H, d, *J* = 8.5 Hz, Ar-H), 7.42-7.49 (2H, br, 2NH), 7.52 (1H, br, NH). EI-MS: m/z ($C_{13}H_{19}N_5O_4$): 323 [M^+]. Analysis calcd. for $C_{13}H_{19}N_5O_4$: C, 50.48; H, 6.19; N, 22.64. Found: C, 50.31; H, 6.12; N, 22.55.

***Sugar N'*-(2-((4-substituted-aryl)amino)acetyl) succinohydrazone (13-15).**

A solution of the hydrazide derivative **10-12** (10 mmol) in ethanol (20 mL) was added to D-xylose (10 mmol) suspended in water (1 mL) followed by a catalyzed amount of glacial acetic acid (3-5 drops). The reaction mixture was heated under reflux for 6-9 h as estimated by TLC then cooled to room temperature. The formed precipitate was filtered, washed with cold ethanol then dried and crystallized from ethanol to produce the corresponding sugar hydrazone product 13-15, respectively.

***N'*^{1}-(2-((4-Bromophenyl)amino)acetyl)-*N'*^{4}-((*2S,3R,4R*)-2,3,4,5-tetrahydroxy-pentylidene)succinohydrazide (13).**

Yield: 76%; m.p. 188-189 °C; IR (KBr) cm^{-1}: 3424-3420 (OH), 3384 (NH), 3205 (NH), 3045 (CH-arom.), 2923 (CH-aliph), 1693 (C=O), 1632 (C=O), 1490 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 2.40-2.47 (2H, 4H, 2CH_2), 3.36-3.39 (2H, m, H-5',5"), 3.71 (3H, m, H-4', CH_2), 3.95-3.98 (m, 1H, H-3'), 4.15-4.21 (m, 3H, H-2', OH), 5.02-5.10 (4H, m, OH, NH, NH_2), 5.12 (m, 2H, OH), 6.02 (br, 1H, NH), 7.11 (2H, d, *J* = 8.5 Hz, Ar-H), 7.24 (2H, d, *J* = 8.5 Hz, Ar-H), 7.94 (d, 1H, *J* = 8.8 Hz, H--1'), 8.56 (2H, br, 2NH). Analysis calcd. for $C_{17}H_{24}BrN_5O_7$: C, 41.64; H, 4.93; N, 14.28. Found: C, 41.41; H, 4.98 N, 14.19.

***N′¹*-((*2S,3R,4R*)-2,3,4,5-Tetrahydroxypentylidene)-*N′⁴*-(2-(*p*-tolylamino)-acetyl)succinohydrazide (14)**.

Yield: 79%; m.p. 192-193 °C; IR (KBr) cm^{-1}: 3369-3355 (OH), 3342 (NH), 3282 (NH), 3048 (CH-arom), 2962 (CH-aliph), 1683 (C=O), 1490 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 2.38 (3H, 2, CH_3), 2.46-2.52 (4H, m, $2CH_2$), 3.32-3.42 (4H, m, H-5',5", CH_2), 3.52-3.55 (1H, m, H-4'), 3.85 (1H, m, H-3'), 4.33 (2H, m, H-2', OH), 4.90 (1H, m, OH), 5.12-5.16 (2H, m, NH, OH), 7.17 (1H, d, *J* = 8.5 Hz, Ar-H), 7.19 (1H, d, *J* = 8.5 Hz, Ar-H), 7.51 (1H, d, *J* = 8.8 Hz, H-1'), 8.42 (2H, br, 2NH), 8.89 (1H, s, NH). ^{13}C NMR (DMSO-d_6) ppm, *δ* 20.5 (CH_3), 30.2, 41.1, 61.7 (3 CH_2), 67.5 (C-5), 68.3 (C-4), 70.4 (C-3), 71.4 (C-2), 115.7 -146.5 (Ar-C), 135.3 (C-1), 169.6, 169.8, 169.9 (3 C=O). Analysis calcd. for $C_{18}H_{27}N_5O_7$: C, 50.82; H, 6.40; N, 16.46. Found: C, 51.03; H, 6.31; N, 16.35.

***N′¹*-(2-((4-Methoxyphenyl)amino)acetyl)-*N′⁴*-((*2S,3R,4R*)-2,3,4,5-tetra-hydroxypentylidene) succinohydrazide (15).**

Yield: 75%; m.p. 185-186 °C; IR (KBr) cm-1, 3423-3410 (OH), 3095 (NH), 3004 (CH-arom), 2925 (CH-aliph), 1637 (C=O), 1491 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 2.40 (2H, t, *J* = 6.2 Hz, CH_2), 2.48 (2H, t, *J* = 6.2 Hz, CH_2), 3.41-3.47 (2H, m, H-5',5"), 3.70 (3H, s, OCH_3), 3.74 (1H, s, CH_2), 3.79 (1H, m, H-4'), 4.33 (1H, m, H-3'), 4.91 (2H, m, H-2', OH), 4.97 (m, 1H, OH), 5.12 (2H, m, OH, NH), 7.16 (1H, d, *J* = 8.5 Hz, Ar-H), 7.17 (1H, d, *J* = 8.5 Hz, Ar-H), 7.52 (1H, d, *J* = 8.8 Hz, H-1') 8.41 (2H, br, NH), 8.88 (1H, br, NH). Analysis calcd. for $C_{18}H_{27}N_5O_8$: C, 48.98; H, 6.17; N, 15.87. Found: C, 48.71; H, 6.11; N, 15.97.

(*2R,3R,4S*)-5-(2-(4-(2-(2-((4-Bromophenyl)amino) acetyl)hydrazinyl)-4-oxobutanoyl)hydrazono)pentane-1,2,3,4-tetrayl tetraacetate (16-18)..

A solution of sugar hydrazone 13-15 (2 mmol) in pyridine (10 mL) was added, acetic anhydride (3 mL) was added and the reaction mixture was stirred at room temperature overnight. After all the amount of starting material is consumed, poured the content into ice-cold water. The product was extracted by ethyl acetate and the organic layer was cleaned with water (2 × 30 mL) soaked in sodium hydrogen carbonate and dried over anhydrous sodium solution. Evaporation of the solvent conferred the sugar hydrazones per *O*-acetyl **16-18**, respectively.

***2R,3R,4S*)-5-(2-(4-(2-(2-((4-Bromophenyl)amino)acetyl)hydrazinyl)-4-oxobutanoyl)hydrazono)pentane-1,2,3,4-tetrayl tetraacetate (16)**.

Yield: 70%; Brownish foam; IR (KBr) cm^{-1}: 3421 (NH), 3001 (CH-arom), 2927-2854 (CH-aliph), 1631 (C=O), 1511 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 1.97, 1.99, 2.03, 2.05 (12H, 4s, $4CH_3$), 2.47 (2H, t, *J* = 6.2 Hz, CH_2), 2.80 (2H, t, *J* = 6.2 Hz, CH_2), 3.55 (2H, s, CH_2), 3.92-3.95 (m, 1H, H-5'), 4.03-4.05 (dd, 1H, *J* = 3.4, 10.8 Hz, H-5"), 4.95-5.04 (2H, m, H-4',3'), 5.55 (2H, m, H-2', NH), 7.09 (2H, d, *J* = 8.5 Hz, Ar-H), 7.19 (2H, d, *J* = 8.5 Hz, Ar-H), 7.52 (1H, d, *J* = 8.8 Hz, H-1'), 8.12 (2H, br, 2NH), 9.12 (1H, br, NH). ^{13}C NMR (DMSO-d_6) ppm, *δ* 20.3, 20.7, 20.8, 20.9 (4 CH_3) 37.7, 54.6, 56.7 (3 CH_2), 67.9 (C-5), 70.5 (C-4), 72.5 (C-3), 73.7 (C-2), 107.5 -146.6 (Ar-C), 149.9 (C-1), 168.6, 168.7, 169.7, 169.9, 170.1, 170.2, 170.4 (7 C=O). Analysis calcd. for $C_{25}H_{32}BrN_5O_{11}$: C, 45.60; H, 4.90; N, 10.64. Found: C, 45.47; H, 4.95; N, 10.51.

(*2R,3R,4S*)-5-(2-(4-Oxo-4-(2-(2-(*p*-tolylamino) acetyl)hydrazinyl)butanoyl)-hydrazono)pentane-1,2,3,4-tetrayl tetraacetate (17).

Yield: 67%; Brownish foam; IR (KBr) cm^{-1}: 3258 (NH), 3117 (CH-arom), 2927 (CH-aliph), 1739 (C=O), 1620 (C=O), 1510 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 1.98, 2.00, 2.03, 2.06, 2.22 (15H, 5s, $5CH_3$), 2.48 (2H, t, *J* = 6.2 Hz, CH_2), 2.82 (2H, t, *J* = 6.2 Hz, CH_2), 3.66 (2H, s, CH_2), 3.92-3.95 (m, 1H, H-5'), 4.21-4.24 (dd, 1H, *J* = 3.4, 10.8 Hz, H-5"), 4.95-5.04 (2H, m, H-4',3'), 5.67-2.71 (2H, m, H-2', NH), 7.20 (2H, d, *J* = 8.5 Hz, Ar-H), 7.23 (2H, d, *J* = 8.5 Hz, Ar-H), 7.52 (1H, d, *J* = 8.8 Hz, H-1'), 9.75 (2H, br, 2NH), 9.81 (1H, br, NH). ^{13}C NMR (DMSO-d_6) ppm, *δ* 19.8, 20.4, 20.6, 20.7, 22.3 (5 CH_3) 39.2, 41.2, 61.5 (3 CH_2), 67.8 (C-5), 68.3 (C-4), 70.4 (C-3), 71.5 (C-2), 111.7 -146.6 (Ar-C), 153.5 (C-1), 169.1, 169.3, 169.4, 169.6, 169.8, 171.2 (6 C=O). Analysis calcd. for $C_{26}H_{35}N_5O_{11}$: C, 52.61; H, 5.94; N, 11.80. Found: C, 52.43; H, 6.01; N, 11.92.

(*2R,3R,4S*)-5-(2-(4-(2-(2-((4-Methoxyphenyl)amino)acetyl)hydrazinyl)-4-oxobutanoyl)hydra-zono)pentane-1,2,3,4-tetrayl tetraacetate (18).

Yield: 82%; Brownish foam; IR (KBr) cm^{-1}: 3423 (NH), 3095 (NH), 3004 (CH-arom), 2925 (CH-aliph), 1738 (C=O), 1617 (C=O), 1511 (C=C). ^{1}H NMR (DMSO-d_6) ppm, δ: 1.97, 1.99, 2.02, 2.22 (12H, 4s, $4CH_3$), 2.47 (2H, t, *J* = 6.2 Hz, CH_2), 2.84 (2H, t, *J* = 6.2 Hz, CH_2), 3.65-3.72 (5H, m, CH_3, CH_2), 3.92-3.95 (m, 1H, H-5'), 4.23-4.27 (dd, 1H, *J* = 3.4, 10.8 Hz, H-5"), 4.99-5.09 (2H, m, H-4',3'), 5.70-5.79 (2H, m, H-2', NH), 7.05 (2H, d, *J* = 8.5 Hz, Ar-H), 7.27 (2H, d, *J* = 8.5 Hz, Ar-H), 7.52 (1H, d, *J* = 8.8 Hz, H-1'), 9.80 (2H, br, 2NH), 9.92 (1H, br, NH). Analysis calcd. for $C_{26}H_{35}N_5O_{12}$: C, 51.23; H, 5.79; N, 11.49. Found: C, 51.04; H, 5.71; N, 11.57.

CYTOTOXIC ACTIVITIES

Cell culture, maintenance and subculture.

Human cell lines were purchased from the American Type Culture Collection (ATCC, USA) through VACSERA; human colorectal HCT 116, hepatic HepG2, and breast MCF7 cancer cell lines. They were cultured with Dulbecco's modified Eagle medium (DMEM) 10% fetal bovine serum (FBS) was added. Cells were incubated in humidified 5% carbon dioxide gas at 37 °C to maintain growth.

Cell proliferation by MTT assay.

The percentages of human colorectal HCT 116, hepatic HepG2, and breast MCF7 cancer cells after treatment with different concentrations of synthesized compounds evaluated by the 3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay as previously reported [45], with slight modification. Briefly, after evaluating cell number and viability by the trypan blue dye-based method, human colorectal HCT 116, hepatic HepG2 and MCF7 breast cancer cells (1×10^4 cells/well) were seeded into 96-well plates and then kept overnight for attachment. On the next day, the complete medium was replaced with a new one, and then different concentrations (0, 20, 40, 60, 80, 100 μM) of the synthesized compounds on each cell line were examined. 5-Flurouracil (5-FU), a standard anticancer drug, was used as a positive control and administered in doses (0, 20, 40, 60, 80, 100 μM). Then, cells were allowed to grow for 24 h.

Four hours before the incubation period was completed, 5 mg/ml MTT was added into each well. After the incubation was completed, 100 μl of dimethyl sulfoxide (DMSO) was added to each well and left for 20 min to dissolve the formazan crystals. After the reaction, color evolution was measured at 450 nm using a Bio-Tek microplate reader.

IC_{50} measurement Half-maximal inhibitory concentrations (IC50), which are concentrations that kill 50% of cancer cells, were obtained by plotting percentages of cancer cell viability versus sample concentrations using multinomial concentration-response curve fitting models (Origin Pro 8 software).

Quantitative Reverse Transcription-Polymerase Chain Reaction (QRTPCR):

Total RNA Extraction: Total RNA was extracted from HCT 116, HepG2 and MCF-7 cells at the IC_{50} dose of the proposed treatments using the Invitrogen RNA Purification Kit (Thermo Fisher) according to the manufacturer's protocol.

Conversion of RNA to cDNA:

First-strand cDNA was synthesized with 1 μg of total RNA using a RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. These samples were subsequently frozen at a temperature of -80 °C until use for determination of the expression levels of Bax and Bcl-2 genes using real-time PCR.

Real Time PCR Reactions:

Real-time quantitative PCR was performed on a DT-lite real-time PCR system (English/Russian system) using a MiScript SYBR Green PCR kit purchased from (Qiagen, Valenica, CA, USA), as well as the forward and reverse primers for each gene. The nuclic acid sequences of the forward (F) and reverse (R) primers of the Bax and Bcl-2 genes compared to GAPDH as the housekeeping gene are outlined in (Table 3).

Table 3: Primer sequences

Gene		Primer sequence
Bcl-2	F	5'-CTGCACCTGACGCCCTTCACC-3'
	R	5'-CACATGACCCCACCGAACTCAAAGA-3'
Bax	F	5'-ATGGCTTCTATGAGGCTGAG-3'
	R	5'-CGGCCCCAGTTGAAGTTG-3'
GAPDH	F	5'-GTCTCCTCTGACTTCAACAGCG-3'
	R	5'-ACCACCCTGTTGCTGTAGCCAA-3'

The real-time PCR mixture consisted of 10 μl of SYBR PCR Master Mix, 1 μl of each primer material (400 nM), 1 μl of cDNA, and 7 μl of Rnase-free water for a total volume of 20 μl. Amplification conditions and number of cycles were 95°C for 15 min of initial activation, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 seconds. Melting curves were performed after real-time PCR to demonstrate the specific amplification of the tested genes.

CONCLUSION

The functionalized sugar hydrazones and their per-*O*-acetylated derivatives were successfully synthesized *via* a multistep rout starting with simple aryl hydrazide compounds. The afforded results showed, obviously, the effect of attachment of the different sugar moieties which resulted in enhanced cytotoxic and apoptotic activities against different cancerous cells in this investigation. The per-*O*-acetylated sugar hydrazones were generally shown to be the most active anticancer derivatives. The derivatives incorporating the bromo and methyl substituents in the 4-position of the aryl moiety were the most influencing on the MCF-7 cancer for the compounds of such structures. The presented study showed the effect of sugar attachment and encourage for more future studies against human cancer cell lines.

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