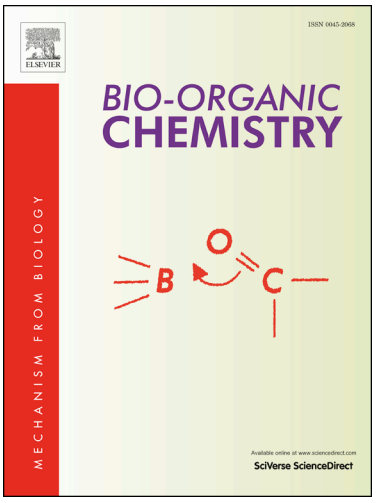

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Synthesis and biological evaluation of benzo[b]furo[3,4-e][1,4]diazepin-1-one derivatives as anti-cancer agents

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Abstract

A new series of novel Podophyllotoxin-like benzo[b]furo[3,4-e][1,4]diazepin-1-ones possessing structural elements of 4-aza-2,3-didehydropodophyllotoxins with central diazepine ring was designed and synthesized as anti-cancer agents. In initial assessment, the cytotoxic activity of the synthesized compounds was evaluated against three cancer cell lines including MCF-7, PC3 and B16-F10 employing the MTT assay. Some of compounds (**12h**, **13a**, **13c** and **14b**) showed significant cytotoxic activity. So, we investigated the cytotoxicity of compounds **12h**, **13a**, **13c** and **14b**, along with podophyllotoxin as the reference drug in different cancer cell lines including A549, A2780, DU145, HeLa, and normal Huvec cell line .Among these four compounds, **13c** showed promising antiproliferative activity against all cancer cells stronger than the other compounds and comparable to reference drug podophyllotoxin in some cancer cells. All these four compounds did not show significant cytotoxicity on normal Huvec cell line. The flow cytometry analysis of the MCF-7, PC3 and A2780 human cancer cell lines treated with **13c**

showed that **13c**, induced apoptosis in the MCF-7, PC3 and A2780 human cancer cell lines, which is in good agreement to its cytotoxic activity as well. Compound **13c** did not show significant influence on tubulin assembly and exert its cytotoxic effects via induction of apoptosis and has potent and selective cytotoxic effects in cancer cells.

Key words; Podophyllotoxin, benzo[b]furodiazepin-1-ones, anti-cancer, cytotoxic, synthesis, apoptosis, tubulin inhibitor

1. Introduction,

Cancer is a major public health problem in the world and it is now the second leading cause of death in the United States, and is predicted to surpass heart diseases as the leading cause of death in the futures [1] Consequently; increasing interest has been devoted to the design and discovery of more effective anticancer agents in current medicinal chemistry [2-9]. Podophyllotoxin (**1**) is the main component of podophyllum resin, a naturally occurring antimitotic cyclolignan which displays strong anticancer activity against numerous cancer cell lines[10]. Although podophyllotoxin possesses notable in vitro antitumor effects, it is not used as an anticancer drug because of its various side effects such as nausea, vomiting and damage of normal tissues. Though it's unfavorable pharmacological profiles, podophyllotoxin can still be used as a lead compound for the discovering of possible anticancer drugs. Etoposide (**2**), teniposide (**3**), and etoposide phosphate (**4**) are semisynthetic derivatives of 4-epipodophyllotoxin. They have numerous limitations, such as poor water solubility, metabolic inactivation, development of drug resistance and toxic effects. To overcome such problems, extensive synthetic efforts have been carried out by a number of researchers. This has led to the development of TOP-53 (**5**) and F14512 (**6**). Recently novel podophyllotoxin derivatives have been reported by researchers [11-17]. 4-aza-2,3-didehydropodophyllotoxins (**7**) have been also reported as anticancer agents [18-21], which displayed cytotoxicity comparable to that of podophyllotoxin. In the present study we report the synthesis of novel podophyllotoxin-like benzo[b]furo[3,4-e][1,4]diazepin-1-ones possessing structural elements of 4-aza-2,3-didehydropodophyllotoxins with central dihydrodiazepine ring (**Fig.1**). The synthesized compounds were initially evaluated for their cytotoxic activity towards three different cancer cell cancer cell lines including MCF-7, PC3 and B16-F10 cancer cells employing the MTT assay. Then we investigated the cytotoxicity of compounds showed the most antiproliferative activity in initial assessment, in different human

cancer cell lines including A549, A2780, DU145, HeLa, and normal Huvec cell line. The effect of the most cytotoxic compounds to induce apoptosis was evaluated by apoptosis assay and compounds **13c** was evaluated for tubulin polymerization inhibitory effect.

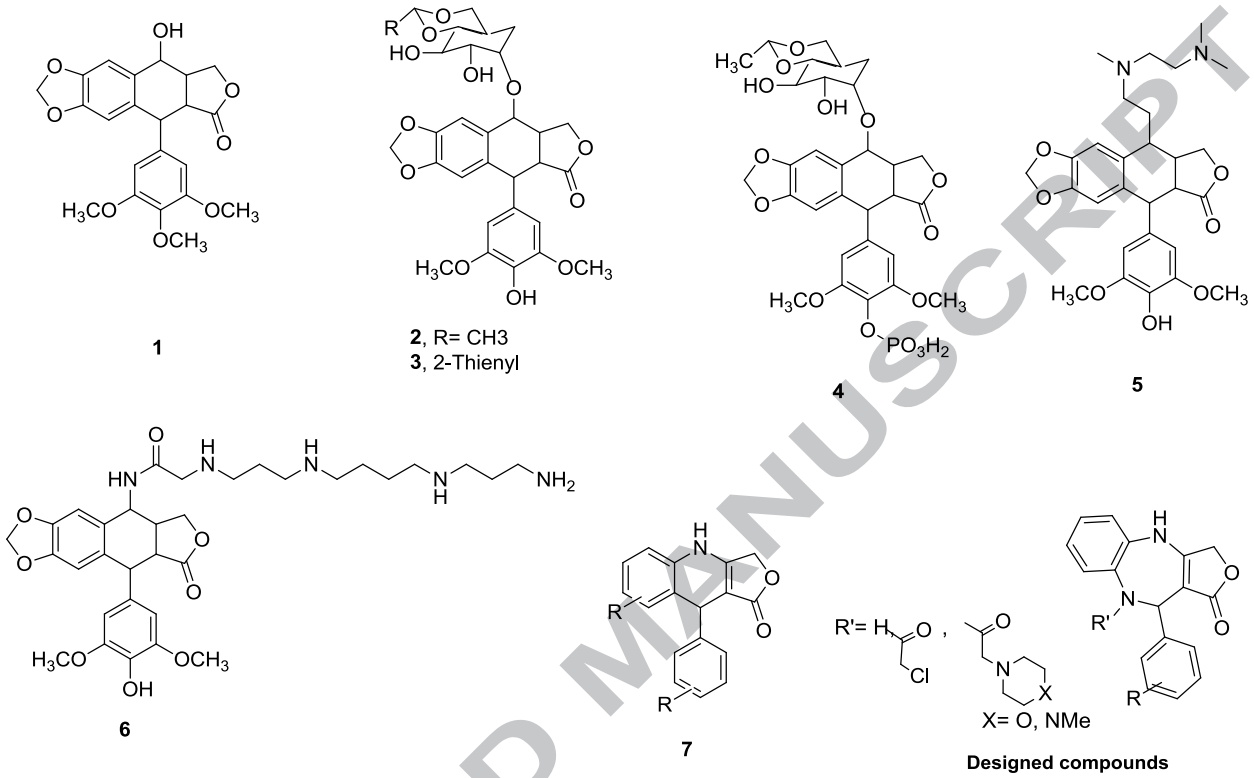


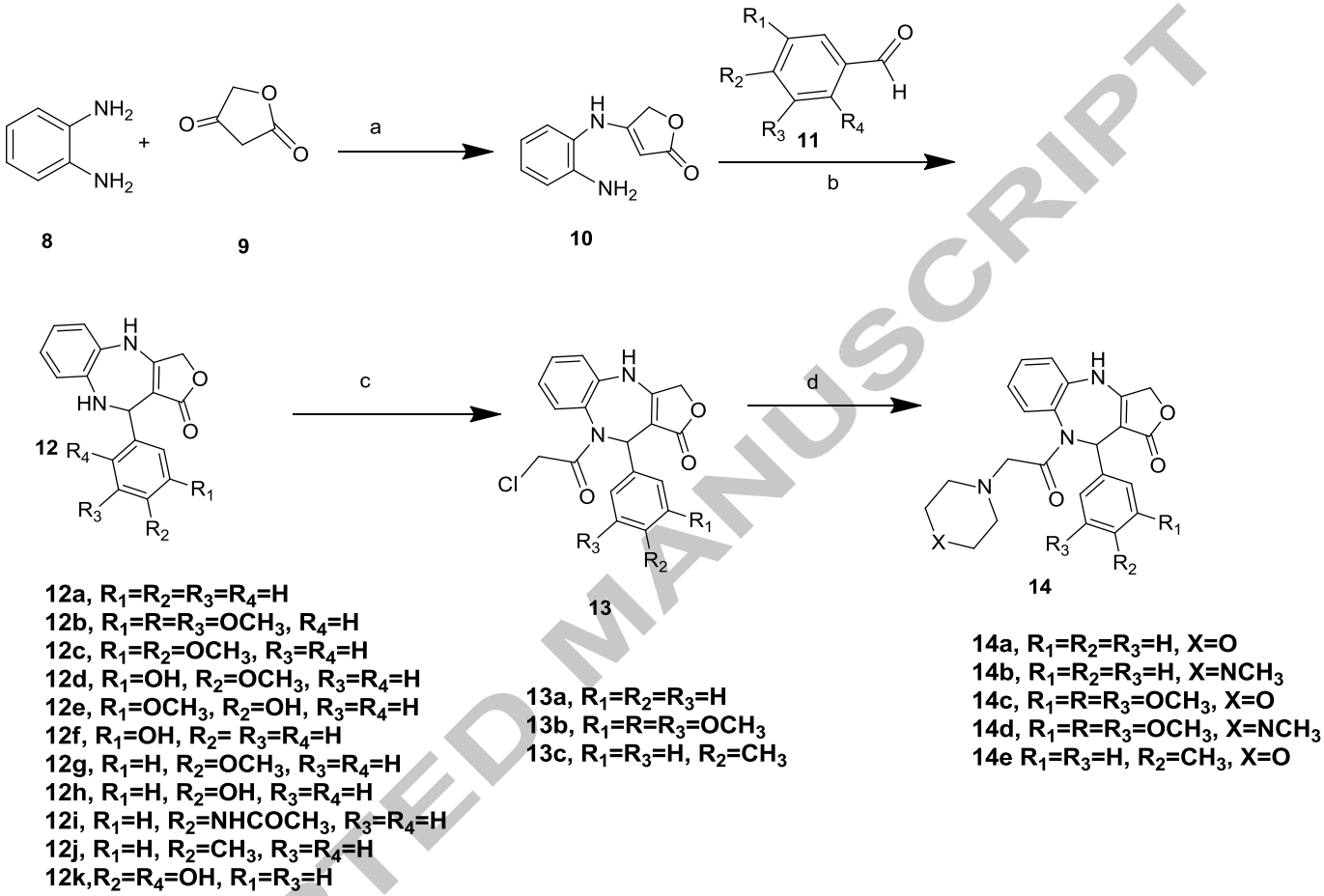
Fig. 1. Structures of podophyllotoxin, its semisynthetic analogs and our designed compounds

2. Results and discussion

2.1. Synthesis

As illustrated in **Scheme 1**, benzene-1,2-diamine **8** and tetronic acid **9** were stirred in ethanol in the presence of catalytic amount of acetic acid to afford 4-((2-aminophenyl)amino)furan-2(5H)-one **10**, then substituted benzaldehydes **11** and compound **10** were dissolved in chloroform and combined in the presence of catalytic amount of acetic acid under microwave irradiation [22] to obtain the target benzo[b]furo[3,4-e][1,4]diazepin-1-ones **12,** then chloroacetyl derivatives **13** were prepared using compounds **12** and chloroacetyl chloride in THF [23]. Reaction of **13** with morpholine or N-methylpiperazine in THF afforded the target compounds **14**. The compounds

were characterized by nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR), infrared and mass spectrometry.



Scheme1. Reagents and conditions; a, EtOH , AcOH, room temperature, 10 h. b, $CHCl_3$, AcOH, Microwave, 30 min, c. Chloroacetyl chloride, THF, reflux. d. Morpholine (N-Methyl piperazine), THF, reflux.

2.2. Biological evaluation

2.2.1. In vitro anticancer activity

The cytotoxic activity of the synthesized compounds was evaluated against MCF-7 (human breast cancer cells), PC3 (human prostate cancer cells), and B16-F10 (Melanoma cells) cancer cells employing the MTT assay. As depicted in **Table 1**, concentrations of 5 and 50 μM of compounds were used for evaluation of cytotoxicity of these compounds. Initial evaluation showed that most of the compounds were not potent cytotoxic agents at concentrations below 50

μM, among the compounds, **13c,** 2-chloroacetyl analog of **12j** possessing *p*-methyl phenyl showed significant cytotoxic activity in all three cancer cells. Compound **12h** possessing *p*-Hydroxy phenyl, **13a**, 2-chloroacetyl analog of **12a** possessing phenyl and **14b,** 2-(4-methylpiperazin-1-yl)acetyl analog of **12a**, also showed cytotoxic activity in cancer cells. As 2-chloroacetyl analogs showed more cytotoxicity in cancer cells compared to 2-morpholinoacetyl and 2-(4-methylpiperazin-1-yl)acetyl analogs, it can be concluded that the higher cytotoxic activity of 2-chloroacetyl analogs could be attributed to their higher lipophilicity. Among the compounds **12a-12k**, Compound **12h** possessing *p*-Hydroxy phenyl was the most cytotoxic agent in MCF-7 and B16-F10 cancer cells. Among the chloroacetyl derivatives **13a-13c**, **13c**, possessing *p*-tolyl, was the most cytotoxic agents. Surprisingly, compound **13b** possessing 3,4,5-trimethoxyphenyl similar to podophyllotoxin, showed the least cytotoxic activity among the series, it might be because of more steric hindrance occurred by three methoxy groups. So it can be concluded that in 2-chloroacetyl analogs, small lipophilic group in *para* position of phenyl ring can increase the cytotoxic activity in cancer cells. Since researchers reported 4-aza-2,3-didehydropodophyllotoxins (**7**) as anticancer agents [18-21], which displayed cytotoxicity comparable to that of podophyllotoxin and more than those of our compounds **12a-12k**, we can conclude that expansion of the central ring of 4-aza-2,3-didehydropodophyllotoxins from six-membered ring (dihydroquinoline) to seven-membered ring (dihydrodiazepine) can decrease the cytotoxic activity of these compounds. Among the 2-morpholinoacetyl and 2-(4-methylpiperazin-1-yl)acetyl derivatives **14a-14e**, **14b,** 2-(4-methylpiperazin-1-yl)acetyl analog of **12a,** showed the highest cytotoxicity. So we investigated the cytotoxicity of compounds **12h**, **13a**, **13c** and **14b** and podophyllotoxin as the reference drug in six cancer cell lines including A549 (adenocarcinoma human alveolar basal epithelial cells), A2780 (human ovarian cancer cells), DU145 (human prostate cancer cells), HeLa (cervical cancer cells), MCF-7 (human breast cancer cells), PC3 (human prostate cancer cells), and normal Huvec cell line (Human Umbilical Vein Endothelial Cells) (**Table 2**) .Among these four compounds, chloroacetyl derivatives **13a** showed significant cytotoxic activity in all cancer cells. Compound **13c** showed promising antiproliferative activity against all cancer cells stronger than the other compounds and comparable to reference drug podophyllotoxin in some cancer cells. All these four compounds did not show significant cytotoxicity on normal Huvec cell line. Compounds 13a and 13c showed potent and selective cytotoxic effects in cancer cells.

Table 1. In-vitro cytotoxicity of compounds (Percent survival of cancer cells (Mean ± SD) following exposure to 5 μM and 50 μM concentrations of compounds)

Compound	**PC3 (5μM)**	**PC3 (50μM)**	**MCF-7 (5μM)**	**MCF-7 (50μM)**	**B16F10 (5μM)**	**B16F10 (50μM)**
12a	103.83±3.51	104.001±5.43	87.01±3.04	65.45±9.07	99.28±6.61	54.94±0.27
12b	99.10±7.16	99.82±7.41	97.31±15.95	79.71±2.07	104.97±13.35	60.80±6.43
12c	107.24±15.16	73.42±16.35	64.61±2.31	**46.12±7.06**	97.45±4.46	**59.62±3.69**
12d	101.07±3.60	102.15±7.93	91.36±12.72	74.28±1.67	99.05±8.39	94.84±4.79
12e	96.88±1.26	80.43±5.56	99.48±14.85	68.58±4.95	101.18±5.04	98.163±5.16
12f	103.47±7.21	95.33±6.89	86.69±4.86	89.89±6.161	99.46±5.88	**58.08±0.93**
12g	97.78±7.80	102.03±6.58	104.9±8.25	95.20±11.44	96.68±4.90	77.91±5.91
12h	102.87±9.03	**54.85±6.58**	90.65±3.62	62.14±8.28	97.21±0.87	**18.65±9.26**
12i	96.82±4.39	96.221±5.91	103.32±1.27	**59.11±2.18**	97.63±8.37	100.11±20.60
12j	90.90±14.36	63.432±4.55	88.03±7.29	86.30±4.13	103.61±5.64	63.52±17.22
12k	100.95±15.80	94.37±1.97	89.89±1.81	90.33±4.42	98.93±2.98	100.41±5.19
13a	61.91±4.28	**6.28±4.69**	86.18±5.49	10.28±2.29	89.63±10.40	**40.55±7.22**
13b	92.69±4.76	71.27±8.89	78.82±16.65	86.75±4.22	90.34±11.82	70.21±6.87
13c	**58.88±2.27**	**11.31±3.68**	**54.44±13.06**	**1.60±0.38**	**27.53±6.60**	**6.92±3.67**
14a	101.19±5.29	88.98±14.7	87.46±5.96	**47.02±4.10**	105.62±5.44	99.23±11.17

		5				
14b	86.65±8.01	**56.13±14.55**	86.88±7.77	**44.01±13.54**	105.38±7.14	105.62±11.29
14c	93.77±3.37	102.03±8.76	105.50±1.17	77.28±11.31	87.68±10.62	99.58±7.65
14d	97.06±4.03	80.67±8.11	97.31±15.67	83.74±6.51	98.34±5.16	98.22±6.46
14e	99.64±6.37	100.172±2.99	105.18±11.14	82.46±6.05	97.75±7.19	95.14±3.08
podophyllotoxin	19.45±3.64	ND	15.62±2.84	ND	ND	ND

ND: not determined

Table 2. The in vitro anti-proliferative activities (**IC_{50} (μM))** of compounds, and podophyllotoxin against human cancer cell lines and normal Huvec cell line

Compound	A549	A2780	DU145	PC3	HeLa	MCF-7	Huvec
12h	>50	>50	>50	48.34±4.12	20.27±2.04	>50	>100
13a	20.38±1.02	5.1±1.01	3.9±0.42	7.13±2.17	10.43±2.02	12.64±1.03	50.17±3.04
13c	10.46±1.34	2.72±0.33	3.89±0.45	6.19±2.65	3.51±1.21	9.37±2.07	44.73±2.44
14b	48.53±3.82	>50	>50	45.67±3.87	>50	46.23±5.24	>100
podophyllotoxin	1.64±0.16	0.86±0.25	0.97±0.13	1.26±0.17	1.21±0.14	1.02±0.13	1.82±0.17

IC_{50}: compound concentration required to inhibit tumor cell proliferation by 50%. Data are presented as the mean ± SD from the dose–response curves of three independent experiments

2.2.2. Apoptosis assay

2.2.2.1. PI staining

To investigate whether compound **13c** and **14b** induce apoptosis of cells, the effect of **13c** and **14b** to induce apoptosis was evaluated by apoptosis assay with flow cytometry using PI staining test [24]. Cells were treated with 5μM and 50μM concentrations of **13c** and **14b** for 48 h. the percentage of sub-G1 apoptotic cells in **13c** and **14b** treatments was determined by flow cytometry. As shown in **Fig. 2**, the results proved that **13c**, in addition to its anti-proliferative activity, also induced apoptosis in MCF-7 and PC3 cancer cell lines, which is in good agreement to its cytotoxic activity as well. The percentage of apoptotic cells induced by **13a** in MCF-7 cells increased from 10.5% (control) to 33% (5 μM) and 83.9% (50 μM) respectively and the percentage of apoptotic cells induced by **14b** increased from 10.5% (control) to 14.6% (5 μM) and 18.5% (50 μM), respectively. Cell cycle analysis showed that the ratio of apoptosis in

PC3cells for compound **13c** at 5 and 50 µM was 71% and 83.73% respectively, and for compound **14b** at 5 and 50 µM it was found to be 26.1% and 23.9% respectively.

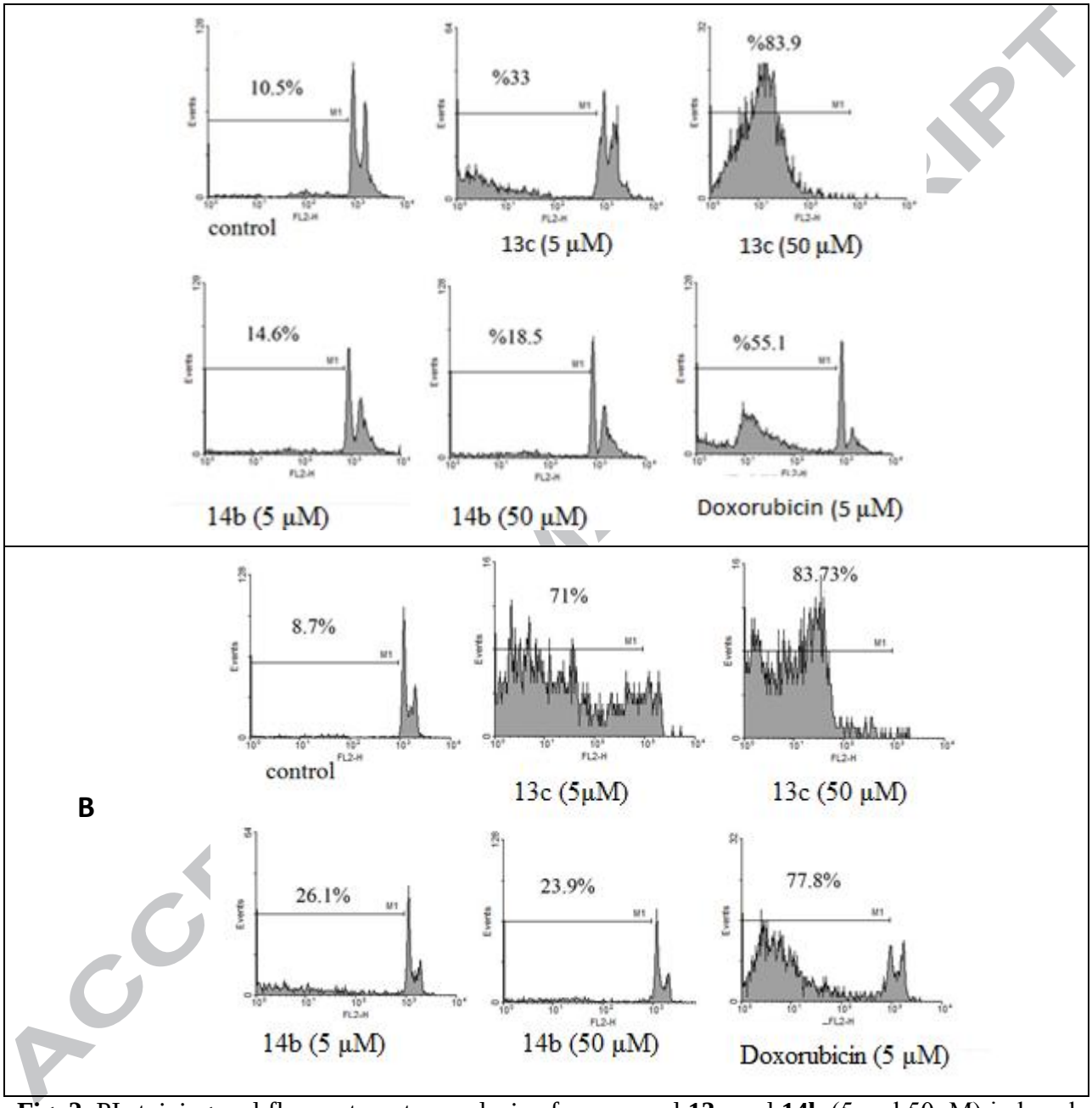


Fig. 2. PI staining and flow cytometry analysis of compound **13c** and **14b** (5 and 50µM) induced apoptosis (**A**) in MCF-7 cells and (**B**) in PC3 cells.

2.2.2.2. Apoptosis assay (Annexin V binding staining)

As compounds **13a** and **13c** showed the most cytotoxic effect in A2780 cancer cells, to quantify the percentage of cells undergoing apoptosis by compound **13a** and **13c**, Annexin V-

FITC/Propidium iodide staining assay was performed in A2780 cancer cells according to reported procedures [25]. This assay enables the detection of live cells (Q4), early apoptotic cells (Q3), late apoptotic cells (Q2) and necrotic cells (Q1). A2780 cells were treated with 10 μM, and 50 μM concentrations of compounds and stained with Annexin V-FITC/Propidium iodide. Results from **Fig. 3** showed that the percentage of total apoptotic cells (early and late apoptotic cells) induced by **13a** increased from 0.224% (control) to 21.14% (10 μM) and 76.16% (50 μM) respectively and the percentage of total apoptotic cells induced by **13c** increased from 0.224% (control) to 76.84% (10 μM) and 78.73% (50 μM) respectively. Therefore, the percentage increase in early and late apoptotic cells induced by **13a** and **13c** indicates that these compounds induced apoptosis in A2780 cancer cells.

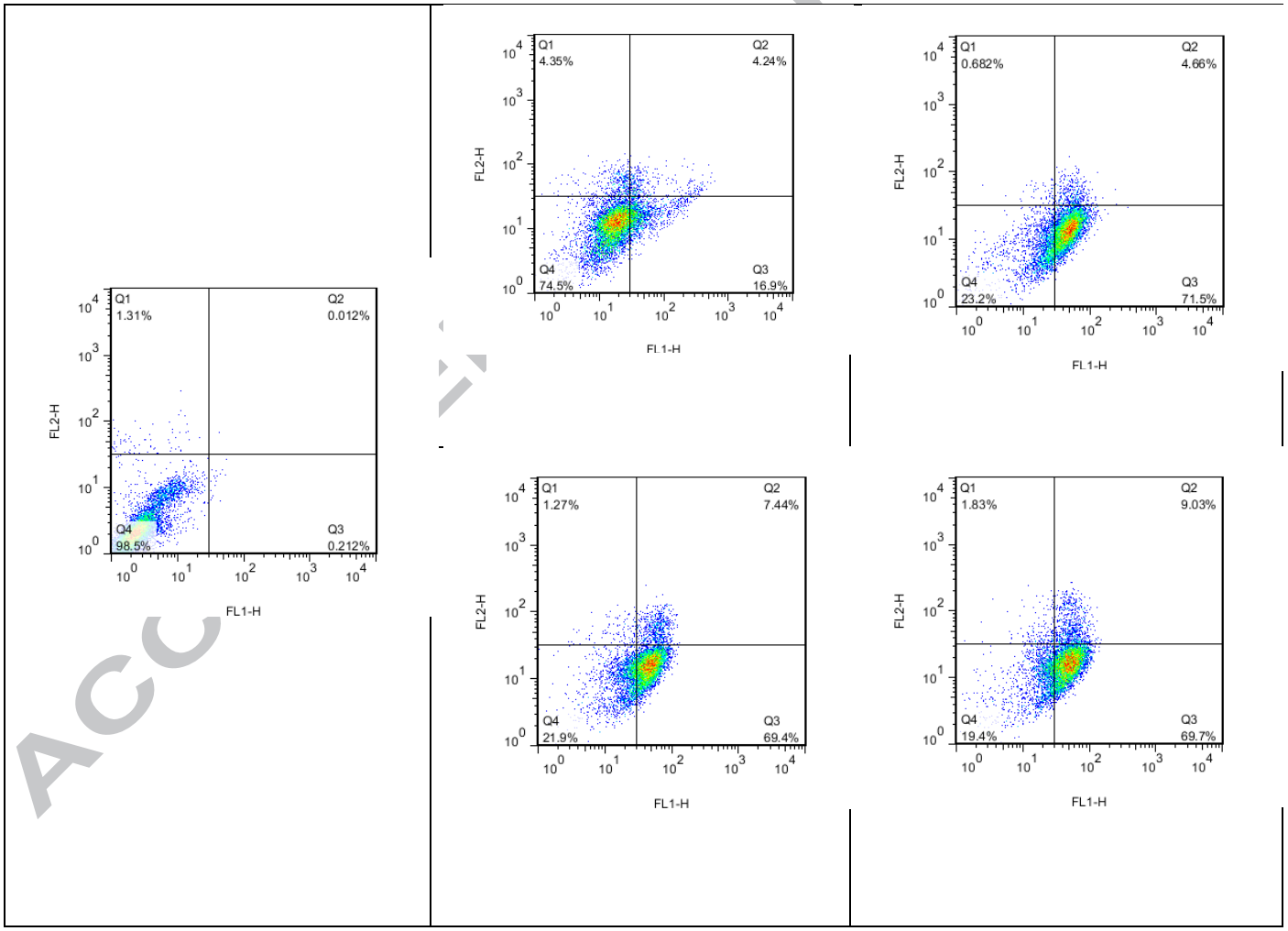


Fig. 3. Effects of **13a** and **13c** on the apoptosis of A2780 cancer cells using Annexin V/PI double staining test by flow cytometry.

2.2.3. Tubulin polymerization assay

To investigate whether the cytotoxic activity of compound **13c** was related to the binding to tubulin, compounds **13c** and reference compounds podophyllotoxin (polymerization suppressor) and a polymerization promoter (paclitaxel) were evaluated for tubulin polymerization inhibitory effect. Compound **13c** which was most cytotoxic compound did not show significant influence on tubulin assembly in comparison to the reference compounds podophyllotoxin (**Fig. 4**). This may be explained by other mechanisms involved in cytotoxicity other than tubulin inhibition.

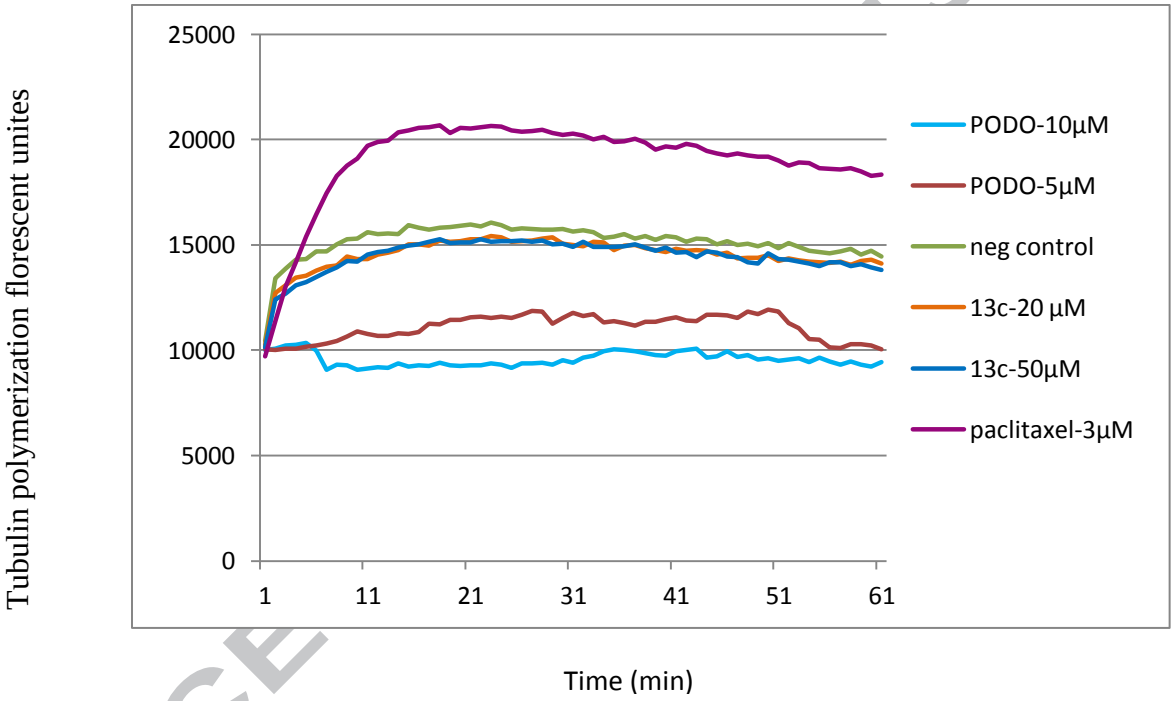


Fig. 4. Effect of compounds (**13c**, podophyllotoxin and Paclitaxel) on in vitro tubulin polymerization

3. Conclusions

A new series of novel Podophyllotoxin-like like benzo[b]furo[3,4-e][1,4]diazepin-1-one analogues was synthesized and evaluated for their cytotoxic activity against different cancer cell lines. Some of compounds (**12h**, **13a**, **13c** and **14b**) showed significant cytotoxic activity. Among these four compounds, **13c** showed promising antiproliferative activity against all six

cancer cell lines including A549, A2780, DU145, HeLa, MCF-7 and PC3, stronger than the other compounds (with IC_{50} in the range of 2.72-10.46 μM) and comparable to reference drug podophyllotoxin in some cancer cells. All these four compounds (**12h**, **13a**, **13c** and **14b**) did not show significant cytotoxicity on normal Huvec cell line. The flow cytometry analysis of the MCF-7 and PC3 and A2780 human cancer cell lines treated with **13c** showed that **13c**, induced apoptosis in the MCF-7, PC3 and A2780 human cancer cell lines, which is in good agreement to its cytotoxic activity as well. We conclude that compound **13c** exert its cytotoxic effects via induction of apoptosis and has potent and selective cytotoxic effects in cancer cells. The data resulted from compound**13c** seems promising and have prompted further evaluation of this compound.

4. Experimental section

4.1. Chemistry

All chemicals, reagents and solvents used in this study were purchased from Merck AG and Aldrich Chemical. Melting points were determined with a Thomas–Hoover capillary apparatus. Infrared spectra were acquired using a Perkin Elmer Model 1420 spectrometer. Bruker FT-400 and 300 MHz instruments (Brucker Biosciences, USA) were used to acquire ^{1}HNMR spectra and A Bruker FT-300 MHz instrument was used to acquire ^{13}CNMR spectra with TMS as internal standard. Chloroform-D and DMSO-D6 were used as solvents. Coupling constant (J) values are assessed in hertz (Hz) and spin multiples are given as s (singlet), d (double), t (triplet), q (quartet), m (multiplet). The mass spectral measurements were performed on a 3200 QTRAP LCMS triple quadrupole mass spectrometer (LCMS) with an electrospray ionization (ESI) interface.

4.2. preparation of 4-((2-aminophenyl)amino)furan-2(5H)-one[23]

A solution of 1 g of benzene-1,2-diamine **8** (10mmol) and 1.084 g tetronic acid **9** were stirred in 10 ml ethanol and acetic acid (0.25 ml) for 10 hours, then the produced precipitate was filtered and washed with ethanol and recrystallized in ethanol to obtain 1g of **10**.

Yield: 53%; mp= 225-227 °C; IR (KBr): v(cm^{-1})3403-3231(NH), 1676-1613(C=O); ^{1}H NMR(400 MHz, DMSO-d6): δ(ppm) 8.77 (s, 1H, NH), 7.05-6.56 (m, 4H, ArH), 5.04-5.02 (d, 1H, NH2, J=7.2 Hz), 4.82 (s, 2H, CH2), 4.55 (s, 1H, CH).

4.3. General procedure for preparation of 10- substituted phenyl-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12a-12j)

4-((2-aminophenyl)amino)furan-2(5H)-one **10** (2.1 mmol), appropriate benzaldehyde **11** (2.1 mmol), acetic acid (0.3 ml) and chloroform (5 ml) were mixed. The mixture was subjected to microwave irradiation at 200 W. Upon completion, monitored by TLC, the reaction mixture was cooled to room temperature then produced precipitate was filtered and washed with chloroform and hexane to obtain the pure compounds.

4.3.1. 10-phenyl-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12a)[22]

Yield: 65%; mp= 200-202 °C; IR (KBr): v(cm^{-1})3352-3201(NH), 1717-1669(C=O); ^{1}H NMR(400 MHz, DMSO-d6): δ(ppm) 9.84 (s, 1H, NH), 7.20-7.08 (m, 5H, ArH), 6.91-6.89 (d,1H, ArH, J=7.6 Hz), 6.75-6.66 (m, 2H, ArH), 6.60-6.58 (d, 1H, ArH, J=7.2 Hz), 6.08-6.07 (d, 1H, NH, J=4.2 Hz), 5.07-5.05 (d, 1H, CH, J=4.4 Hz), 4.90 (s, 2H, CH_2).

4.3.2. 10-(3,4,5-trimethoxyphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12b)[22]

Yield: 52%; mp= 259-260 °C; IR (KBr): v(cm^{-1})3429(NH), 1717-1618(C=O); ^{1}H NMR(500 MHz, DMSO-d6): δ(ppm) 9.779 (s, 1H, NH), 6.87-6.85 (m, 1H, ArH), 6.73-6.67 (m, 2H, ArH), 6.62-6.60 (dd, 1H, ArH, J=9.21, J=1.76 Hz), 6.37 (s, 2H, ArH), 5.96-5.95 (d, 1H, NH, J=4 Hz), 4.94-4.93 (d, 1H, CH, J=4.0 Hz), 4.87-4.80 (d, 2H, CH_2, J=15.1 Hz), 3.55 (s, 6H, OCH_3), 3.51 (s, 3H, OCH_3).

4.3.3. 10-(3,4-dimethoxyphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12c)[23]

Yield: 60%; mp= 230-232 °C; IR (KBr): v(cm^{-1})3419(NH), 1720-1621(C=O); ^{1}H NMR (500 MHz, DMSO-d6): δ(ppm) 9.72 (s, 1H, NH), 6.85-6.82 (m, 2H, ArH), 6.69-6.63 (m, 3H, ArH),

6.58-6.56 (m, 1H, ArH), 6.45-6.43 (m, 1H, ArH), 5.96 (s, 1H, NH), 4.95 (s, 1H, CH), 4.86-4.80 (m, 2H, CH_2), 3.59 (s, 3H, OCH3) 3.57 (s, 3H, OCH_3).

10-(3-hydroxy-4-methoxyphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12d)

Yield: 66%; mp= 263-265 °C; IR (KBr): v(cm^{-1})3320(NH), 1711-1616(C=O); ^{1}H NMR (300 MHz, DMSO-d6): δ(ppm)9.84 (s, 1H, NH),), 8.78 (s, 1H, OH), 6.94-6.91 (m, 1H, ArH),6.80-6.71 (m, 3H, ArH), 6.66-6.61 (m, 2H, ArH), 6.58-6.55 (m, 1H, ArH), 5.96-5.95 (d, 1H, NH, J=4.4 Hz), 4.95 (s, 1H, CH), 4.92 (s, 2H, CH_2), 3.72 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 55.98, 57.27, 66.44, 97.61, 112.07, 115.27, 118.34, 119.89, 120.99, 123.29, 131.89, 136.95, 137.96, 146.27, 146.72, 159.00, 173.30. LC-MS (ESI): 325.1 (M+1).

10-(4-hydroxy-3-methoxyphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12e)

Yield: 67%; mp= 252-254 °C; IR (KBr): v(cm^{-1})3241(NH), 1725-1622(C=O); ^{1}H NMR (400 MHz, DMSO-d6): δ(ppm)9.77 (s, 1H, NH),), 8.75 (s, 1H, OH),), 6.84-6.61 (m, 7H, ArH), 5.97 (s, 1H, NH), 4.96 (s, 1H, CH), 4.87 (s, 2H, CH_2), 3.62 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 55.89, 57.54, 66.49, 97.56, 112.26, 115.07, 119.53, 119.86, 121.15, 123.31, 123.47, 132.18, 135.26, 138.13, 145.60, 147.71, 159.27, 173.41. LC-MS (ESI): 325.1 (M+1).

10-(3-hydroxyphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12f)

Yield: 71%; mp= 280-282 °C; IR (KBr): v(cm^{-1})3636(OH), 3319(NH), 1714-1647(C=O); ^{1}H NMR(500 MHz, DMSO-d6): δ(ppm)9.74 (s, 1H, NH), 9.11(s,1H,OH), 6.91 (t, 1H, ArH), 6.84-6.82 (m, 1H, ArH), 6.68-6.64 (m, 2H, ArH), 6.56-6.52 (m, 2H, ArH), 6.46-6.44 (m, 2H, ArH), 5.96-5.95 (d, 1H, NH, J=4.5 Hz), 4.899-4.890 (d, 1H, CH, J=4.5 Hz), 4.85-4.79 (d, 2H, CH_2, J=15.5 Hz); ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 57.68, 66.48, 97.26, 113.90, 114.68, 118.41, 119.94, 121.01, 123.24, 123.32, 129.33, 131.75, 137.82, 145.70, 157.43, 159.06, 173.30. LC-MS (ESI): 295.0 (M+1).

4.3.4. 10-(4-methoxyphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12g)

Yield: 63%; mp= 222-224 °C; IR (KBr): v(cm^{-1})3563-3417(NH), 1718-1667(C=O); ^{1}H NMR (400 MHz, DMSO-d6): δ(ppm) 9.80 (s, 1H, NH), 7.06-7.03 (m, 2H, ArH), 6.89-6.87 (m, 1H, ArH), 6.75-6.68 (m, 4H, ArH), 6.608-6.604 (m, 1H, ArH), 5.96-5.95 (d, 1H, NH, J=4.4 Hz), 5.02-5.01 (d, 1H, CH, J=3.2 Hz), 4.88 (m, 2H, CH_2), 3.65 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 55.35, 57.26, 66.56, 97.52, 113.79, 119.98, 121.20, 123.37, 123.40, 128.68, 132.10, 136.29, 137.86, 158.35, 159.23, 173.36. LC-MS (ESI): 309.0 (M+1).

4.3.5. 10-(4-hydroxyphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12h)

Yield: 73%; mp= 263-265 °C; IR (KBr): v(cm^{-1})3377(OH), 3235(NH), 1729-1617(C=O); ^{1}H NMR(400 MHz, DMSO-d6): δ(ppm) 9.75 (s, 1H, NH), 9.15(s,1H,OH), 6.92-6.90(d,2 H, ArH,J=8.4 Hz), 6.87-6.85 (d, 1H, ArH, J=1.6 Hz), 6.76-6.65 (m, 2H, ArH), 6.58-6.56, (d, 1H, ArH, J=1.6 Hz), 6.55-6.53 (d, 2H, ArH, J=8.4Hz), 5.93-5.92 (d, 1H, NH, J=4.4 Hz), 4.96-4.95(d, 1H, CH,J=4.4 Hz), 4.86(s, 2H, CH_2) ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 57.31, 66.51, 97.67, 115.188, 119.92, 121.13, 123.31, 123.42, 128.64, 132.11, 134.64, 137.97, 156.400, 159.18, 173.38. LC-MS (ESI): 295.0 (M+1).

4.3.6. 10-(4-acetamidophenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12i)

Yield: 65%; mp > 300 °C; IR (KBr): v(cm^{-1})3316, 3281(NH), 1705-1690(C=O); ^{1}H NMR (400 MHz, DMSO-d6): δ(ppm)9.67 (s, 2H, NH), 7.17-6.51 (m, 8H, ArH), 5.96-5.95 (d, 1H, NH, J=4.4 Hz), 4.75 (s, 2H, CH_2), 4.25 (s, 1H, CH), 1.85 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 19.036, 56.54, 66.54, 97.20, 119.12, 119.94, 121.20, 123.34, 123.39, 127.80, 132.12, 137.81, 138.21, 138.84, 159.35, 168.58, 173.32. LC-MS (ESI): 336.0 (M+1).

4.3.7. 10-(p-tolyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12j) [22]

Yield: 67%; mp: 290-292 °C.

4.3.8. 10-(2,4-dihydroxyphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (12k)

Yield: 81%; mp = 291-292 °C; IR (KBr): v(cm^{-1})3295-3115(NH), 1719-1655(C=O); ^{1}H NMR (400 MHz, DMSO-d6): δ(ppm)9.74-9.71 (m, 2H, OH),9.01(s, 1H, NH), 6.86-6.84 (m, 1H, ArH), 6.76-6.59 (m, 2H, ArH), 6.50-6.48 (m, 1H, ArH), 6.32-6.28(m, 2H, ArH), 5.89-5.86(m, 1H, ArH), 5.96-5.95 (d, 1H, NH, J=4.4 Hz), 5.03 (s, 1H, CH), 4.90-4.88 (d, 2H, CH2, J=5.2 Hz). ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 53.46, 66.57, 96.82, 102.91, 105.71, 116.84, 120.41, 121.47, 123.27, 123.41, 127.65, 132.18, 137.91, 156.48, 157.70, 160.28, 173.30. LC-MS (ESI): 311.0 (M+1).

4.4. General procedure for preparation of 9-(2-chloroacetyl)-10-substituted phenyl-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (13a-13c)

Chloroacetyl chloride (2.2 mmol) was added to a suspension of compound **12 (**2 mmol) in dry THF (30 mL). The mixture was refluxed with stirring for 3 h. On cooling, the precipitate that formed was filtered off and washed with hexane and ether to give compound **13.**

4.4.1. 9-(2-chloroacetyl)-10-phenyl-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1- one (13a)

Yield:79%; mp = 165-167 °C; ^{1}H NMR (300 MHz, DMSO-d6): δ(ppm) 3.76-3.81 (d, 1H, CH_2Cl, J=13.5 Hz), 4.20-4.24 (d, 1H, CH_2Cl, J=13.5 Hz), 5.01 (m, 2H, CH_2), 6.82-6.86 (m, 2H, CH & ArH), 6.92-6.95 (dd, 1H, ArH, J=8.1, J= 1.2 Hz), 7.05-7.26 (m, 7H, ArH), 10.22(s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 56.10, 67.49, 96.63, 120.77, 120. 85, 123.67, 127.83, 128.08, 128.47, 128.73, 129.87, 132.39, 138.83, 138.89, 138.93, 159.76, 165.59, 172.89. LC-MS (ESI): 355.1 (M+1).

4.4.2. 9-(2-chloroacetyl)-10-(3,4,5-trimethoxyphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (13b)

Yield:75%; mp = 256-258 °C ^{1}H NMR (300 MHz, DMSO-d6): δ(ppm) 3.56 (s, 3H, OCH_3), 3.62(s, 6H, OCH_3), 3.75-3.80 (d, 1H, CH_2Cl, J=13.8 Hz), 4.21-4.25 (d, 1H, CH_2Cl, J=13.8 Hz), 4.94-5.06 (dd, 2H, CH_2, J=15.3 Hz), 6.27 (s, 2H, ArH), 6.75 (s, 1H, CH), 6.89-6.94 (dt, 1H, ArH, J= 8.1, J=1.2 Hz), 7.00-7.03 (dd, 1H, ArH, J=7.8, J= 1.2 Hz), 7.11-7.14 (dd, 1H, ArH, J=7.8, J= 1.2 Hz), 7.25-7.31 (dt, 1H, ArH, J= 8.1, J=1.2 Hz), 10.21(s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 43.48, 56.40, 60.44, 66.95, 96.82, 106.14, 120.70, 120.78, 123.78, 127.97,

128, 129.89, 132.44, 134.63, 137.36, 138.89, 138.99, 153.07, 159.65, 159.78, 165.36, 172.88. LC-MS (ESI): 445.1 (M+1).

4.4.3. 9-(2-chloroacetyl)-10-(p-tolyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (13c)

Yield:68 %; mp = 262-264°C ^{1}H NMR (300 MHz, DMSO-d6): δ(ppm) 2.19 (s, 3H, CH_3), 3.75-3.79 (d, 1H, CH_2Cl, J=13.8 Hz), 4.18-4.22 (d, 1H, CH_2Cl, J=13.8 Hz), 4.94-5.06 (dd, 2H, CH_2, J=15.3 Hz), 6.81 (s, 1H, CH), 6.82-6.88 (t, 1H, ArH, J= 7.2 Hz), 6.92-6.94 (m, 3H, ArH), 7.00-7.03 (d, 2H, ArH, J=7.8Hz), 7.084-7.11 (d, 1H, ArH, J=8.1Hz), 7.25-7.31 (t, 1H, ArH, J= 8.1Hz), 10.22(s, 1H, NH). LC-MS (ESI): 369.1 (M+1).

4.5. General procedure for preparation of 9-(2-morpholino or methylpiperazinyl acetyl)-10-(sustitutedphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (14a-14e)

Morpholin or N-methylpiperazin (5 mmol) was added to a suspension of compound **13** (1 mmol) in dry THF (10 mL). The mixture was refluxed with stirring overnight. The solvent was removed under reduced pressure. It was diluted with water and extracted with ethyl acetate. The combined organic phases was washed with saturated solution of sodium bicarbonate and water, dried over Na_2SO_4, and evaporated under reduced pressure. Then the product recrystallized in ethanol.

4.5.1. 9-(2-morpholinoacetyl)-10-phenyl-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (14a)

Yield: 56%; mp = 145-147°C ^{1}H NMR (300 MHz, DMSO-d6): δ(ppm) 1.99-2.05(m, 2H, CH2-morpholine), 2.19-2.23 (m, 2H, CH2-morpholine), 2.80-2.94 (dd, , 2H, $COCH_2$, J= 14.4 Hz), 3.05-3.09 (t, 2H, CH_2-morpholine, J=5.1), 3.77-3.80 (t, 2H, CH2-morpholine, J=5.1),4.89-5.05 (dd, 2H, CH_2, J=15.3 Hz), 6.79-7.22 (m, 10H, CH & ArH), 10.45(s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 43.21, 53.19, 60.73, 63.79, 66.42, 96.75, 120.31, 123.23, 127.80, 128.48, 128.65, 128.92, 129.46, 132.04, 139.26, 139.50, 159.92, 168.95, 173.03. LC-MS (ESI): 406.2 (M+1).

4.5.2. 9-(2-(4-methylpiperazin-1-yl)acetyl)-10-phenyl-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (14b)

Yield: 48%; mp = 155-157°C ^{1}H NMR (300 MHz, DMSO-d6): δ(ppm) 2.05-2.19 (m, 11H, N-methyl piperazine), 2.79-2.93 (dd, 2H, CH_2N, J=14.4 Hz), 4.89-5.04 (dd, 2H, CH_2, J=15.3 Hz), 6.77-7.22 (m, 10H, CH & ArH), 10.15(s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 46.15, 52.71, 54.89, 5524, 60.48, 66.94, 96.85, 120.29, 123.21, 127.79, 128.47, 128.64, 128.90, 129.49, 132.09, 139.14, 139.54, 159.87, 169.13, 173.03. LC-MS (ESI): 419.1 (M+1).

4.5.3. 9-(2-morpholinoacetyl)-10-(3,4,5-trimethoxyphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (14c)

Yield: 61%; mp = 205-207°C ^{1}H NMR (300 MHz, DMSO-d6): δ(ppm) 1.9-2.23 (m, 4H, CH_2-morpholine), 2.78-2.92 (m, 2H, CH_2N), 3.35-3.37 (m, 4H, CH_2-morpholine), 3.56 (s, 3H, OCH_3), 3.62(s, 6H, OCH_3), 4.88-5.04 (dd, 2H, CH_2, J=15.0 Hz), 6.25 (s, 2H, ArH), 6.76(s, 1H, CH), 6.84-6.94 (m, 2H, ArH), 7.08-7.11 (d, 1H, ArH, J=7.8 Hz), 7.19-7.24 (t, 1H, ArH, J= 7.8 Hz), 10.24(s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 53.19, 55.59, 56.36, 60.42, 60.86, 66.81, 96.99, 106.12, 120.12, 123.38, 128.98, 132.02, 135.23, 137.19, 139.14, 139.24, 153.01, 159.77, 168.70, 173. LC-MS (ESI): 496.2 (M+1).

4.5.4. 9-(2-(4-methylpiperazin-1-yl)acetyl)-10-(3,4,5-trimethoxyphenyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (14d)

Yield:54 %; mp = 195-197°C ^{1}H NMR (300 MHz, DMSO-d6): δ(ppm) 2.05-2.21 (m, 11H, N-methyl piperazine), 2.78-2.92 (dd, 2H, CH_2N, J=14.4 Hz), 3.56 (s, 3H, OCH_3), 3.62(s, 6H, OCH_3), 4.87-5.04 (dd, 2H, CH_2, J=15.3 Hz), 6.25 (s, 2H, ArH), 6.77 (s, 1H, CH), 6.84-6.93 (m, 2H, ArH), 7.07-7.09 (d, 1H, ArH, J=6.9 Hz), 7.19-7.24 (t, 1H, ArH, J= 6.9 Hz), 10.19(s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 46, 52.60, 54.81, 56.49, 60.42, 66.82, 96.97, 106.11, 120.13, 123.32, 128.93, 129.59, 132.07, 135.30, 137.18, 139.06, 139.16, 153.01, 159.63, 168.89, 173.00. LC-MS (ESI): 509.3 (M+1).

4.5.5. 9-(2-morpholinoacetyl)-10-(p-tolyl)-3,4,9,10-tetrahydro-1H-benzo[b]furo[3,4-e][1,4]diazepin-1-one (14e)

Yiled:67 %; mp = 233-235°C ^{1}H NMR (300 MHz, DMSO-d6): δ(ppm) 1.99-2.03(m, 2H, CH_2-morpholine), 2.18 (m, 5H, CH_2-morpholine & CH_3), 2.80-2.94 (dd, , 2H, $COCH_2$, J= 14.4 Hz), 3.38-3.40 (m, 4H, CH_2-morpholine), 4.88-5.02 (dd, 2H, CH_2, J=15.3 Hz), 6.78-7.07 (m, 8H, CH

& ArH), 7.15-7.19 (t, 1H, ArH, J= 6.9 Hz), 10.24(s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d6): δ(ppm) 43.23, 53.20, 55.34, 60.73, 63.79, 66.43, 66.99, 99.77, 123.24, 127.80, 128.49, 128.66, 128.92, 129.46, 132.05, 139.28, 139.51, 159.93, 168.97, 173.05; LC-MS (ESI): 420.2 (M+1), 839.4 (2M+1).

4.6. Cytotoxicity assay

4.6.1. General procedure

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) based assay[26] was carried out by seeding 5000 cancer cells per 180 µL RPMI complete culture medium in each well of 96-well culture plates. The day after seeding, culture medium was replaced with medium containing standard anti-tumor agent as well as different concentrations of newly synthesized quinolines and RPMI control (no drug). Cells were then incubated at 37 ºC in 5% Co2 incubator for 48h. Then 25 µL of MTT solution (4mg Ml^{-1}) were added to each well and further incubated at 37 ºC for 3h. At the end of incubation, formazan crystals were dissolved in 100 µL of DMSO and plates were read in a plate reader (Synergy H4, USA) at 540 nm. This experiment was performed in triplicate determination each time.

4.7. PI staining

MCF-7 and PC3 cells were seeded in each well of a 24-well plate and treated with **13c** and **14b** at concentration of 5 µM and 50 µM and Doxorubicin at concentration of 5 µM or vehicle alone (0.05% DMSO) for 48 h. Floating and adherent cells were then harvested and incubated at 4 ◦C overnight in the dark with 750 µL of a hypotonic buffer (50 µg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100) before flow cytometric analysis using a FAC Scan flow cytometer (Becton Dickinson).

4.8. Annexin V binding assay

A2780 cancer cells were seeded in six-well plates for 24h. The medium was then replaced with complete medium containing compound **13a** and **13b**. After 24 h of treatment, cells from the supernatant and adherent monolayer cells were harvested by trypsinization, washed with PBS at 3000 rpm. Then the cells were processed with Annexin V-assay kit (FITC Annexin V Apoptosis Detection Kit, (Cat. No. ab14085, Abcam, UK)) according to the manufacturer's instruction. Further, flow cytometric analysis was performed using a flow cytometer (Becton Dickinson).

4.9. Tubulin polymerization assay

Tubulin polymerization assay were performed by employing a commercial kit (Tubulin Polymerization Assay Kit (Porcine tubulin and Fluorescence based Kit (Cat. No. BK011P, Cytoskeleton, USA)), according to the manufacturer's protocol [15]. Briefly, tubulin protein was suspended in tubulin buffer (80 mM PIPES, 2 mM MgCl2, 0.5 mM EGTA, 1 mM GTP, 60% (v/v) glycerol, PH 6.9) then added to wells on a 96-well plate containing the cytotoxic compounds or vehicle and mixed well. The effect of compound **13c** at 50 and 100 μM concentrations on tubulin polymerization was evaluated. Tubulin polymerization was followed by monitoring the fluorescence enhancement due to the integration of a fluorescence reporter into microtubules as polymerization happens. Polymerization was measured by excitation at 360 nm and emission at 420 nm for 1 h at 1 min intervals in a plate reader (Synergy H4, USA). Paclitaxel at 3 μM concentration and podophyllotoxin at 5 and 10 μM concentrations were used as positive stabilizing and destabilizing controls, respectively.

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Synthesis and biological evaluation of benzo[b]furo[3,4-e][1,4]diazepin-1-one derivatives as anti-cancer agents

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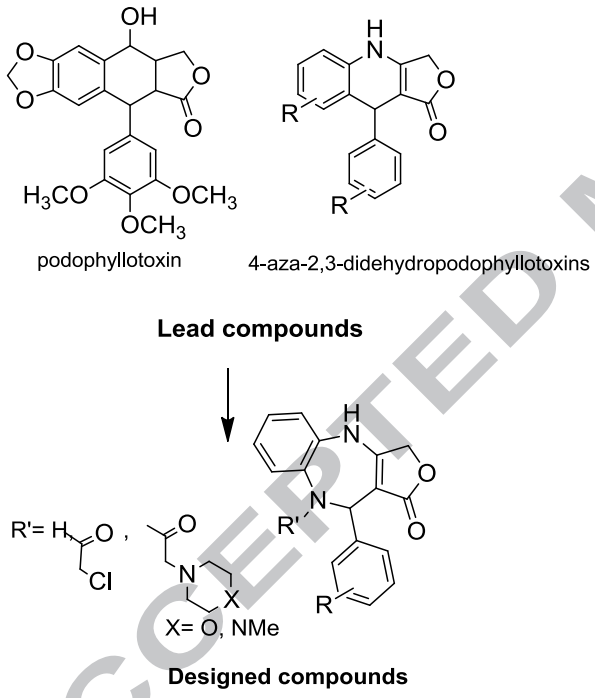

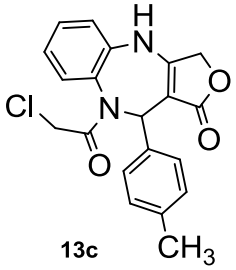

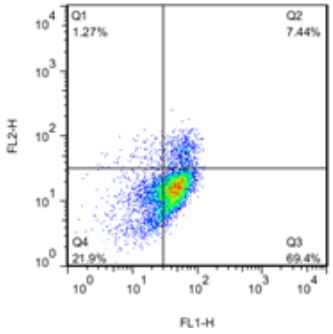

The percentage of total apoptotic cells induced by **13c** increased from 0.224% (control) to 76.84% (10 µM) in A2780 cancer cells.

Compound	A549	A2780	DU145	PC3	HeLa	MCF-7	Huvec
13c	10.46±1.34	2.72±0.33	3.89±0.45	6.19±2.65	3.51±1.21	9.37±2.07	44.73±2.44
podophyllotoxin	1.64±0.16	0.86±0.25	0.97±0.13	1.26±0.17	1.21±0.14	1.02±0.13	1.82±0.17

The in vitro anti-proliferative activities (IC50 (µM)) of **13c**, and podophyllotoxin against human cancer cell lines and normal Huvec cell line

Highlights:

- Novel Podophyllotoxin-like furobenzodiazepines were designed and synthesized as anti-cancer agents
- Some of compounds (**12h**, **13a**, **13c** and **14b**) showed significant cytotoxic activity on cancer cells
- **13c** showed promising antiproliferative activity against all cancer cells stronger than the other compounds.
- The percentage of total apoptotic cells induced by **13c** was 76.84% at concentration of 10 μM