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ORIGINAL ARTICLE

Arylcinnamido-propionone conjugates as tubulin polymerization inhibitors and apoptotic inducers

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Abstract A series of cinnamido-propionone conjugates (**4–6a-h** and **7a-f**) has been designed, synthesized and evaluated for their anticancer potential against some human cancer cell lines. Among them, conjugates **6d** and **6g** have shown significant cytotoxic activity against prostate cancer cells (DU-145) displaying IC_{50} of 7.48 and 8.91 μM respectively. Studies to understand the mechanism of action of **6d** and **6g** indicate that they inhibit the tubulin polymerization thereby arresting the cell cycle in G2/M phase. Furthermore, studies such as mitochondrial membrane potential and Annexin V-FITC assay suggested that these conjugates **6d** and **6g** induced cell death by apoptosis. The molecular docking studies suggested that the binding by these conjugates takes place at the colchicine site of the tubulin protein.
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1. Introduction

Among the current targets for cancer chemotherapy, along-side DNA, microtubules play a critical role (Ducki et al., 2015; Jordan et al., 1998). Microtubules are of particular importance for the formation of the mitotic spindle, which provides the structural framework for the physical segregation of chromosomes during cell division (mitosis) (Desai and Mitchison, 1997; Hyams and Lloyd, 1994; Hadfield et al., 2003). They are crucial in a number of cellular functions, such as cell growth, chromosome segregation during cell division, formation and maintenance of cell shape, regulation of motility, cell signalling and intracellular transport (Amos, 2004; Downing and Nogales, 1998). Microtubules are large, dynamic cylindrical protein copolymers consisting of alternating α and β tubulin heterodimers. Drugs that disrupt microtubule/tubulin dynamics are used widely in cancer chemotherapy interfering with the dynamic instability of microtubules and thereby disrupting microtubules, inducing cell cycle arrest in the M-phase, resulting in the apoptotic cell death (Pasquier et al., 2007). Well known examples include taxanes (such as paclitaxel and docetaxel), that stabilize microtubule by binding to the β−tubulin subunit (Snyder et al., 2001), whereas vinca alkaloids of natural origin and colchicine (**1**), that bind to a different site of β-tubulin and inhibits its assembly into microtubules (Uppuluri et al., 1993). Nocodazole (**2**) is another well-

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known inhibitor of tubulin polymerization which inhibits cell proliferation and largely used for pharmacological tool and positive control (Duanmu et al., 1989; Vasquez et al., 1997). Emerging evidence reveals that targeting tubulin is a promising approach for cancer chemotherapy. However, most of the tubulin-binding agents are derived from natural products with complex chemical structures that restrict chemical modification. Therefore, active compounds with relatively simple chemical structures could be valuable candidates for further development.

Therefore, there is a growing interest in identifying and developing newer molecules that could inhibit tubulin polymerization. Cinnamides are another class of anticancer agents, and its natural analogues are known for the treatment of cancer for over centuries. Phenylcinnamides are shown to bind to tubulin, thereby causing an inhibition of its polymerization and alteration in the tubulin-microtubule equilibrium. They are known to possess an α,β-unsaturated carbonyl moiety, which can be considered as a Michael acceptor, employed as a powerful tool in the design of antimitotic agents and its ability to interact with cellular nucleophiles, particularly to the glutathione (GSH) and cystine residues (Carolin et al., 2014). Hergenrother and co-workers synthesized several phenylcinnamide derivatives to study their structure-activity relationship (SAR) and among them, compound **3 (8H)** showed significant activity (Leslie et al., 2010). These compounds induce M-phase of the cell cycle arrest leading to cell death and disruption of microtubule dynamics. Our recent research studies have been mainly focused on the synthesis, evaluation and mechanistic aspects of newer molecules based on different heterocyclic scaffolds as potential anticancer agents (Kamal et al., 2011, 2012) particularly, tubulin targeting compounds. Some of the potent hybrid/conjugate molecules that have been recently developed as new anticancer agents are obtained by the combination of different pharmacophores (Bonne et al., 1985; Huber et al., 2008). Structural features of designed molecules, including the trimethoxyphenyl moiety (found in colchicine and **8H**), suggested that these molecules exerted cytotoxic action through microtubule binding and mitotic arrest. Based on these observations, we here in describe modifications on **8H** scaffold by conjugating with substituted arylpropynones to the cinnamide moiety. In this context, we have designed and synthesized some newer cinnamido-propionone conjugates and evaluated them for their cytotoxic potential apart from their effect on the inhibition of tubulin polymerization.

2. Material and methods

^{1}H NMR spectra were recorded on Avance 300, Inova 400, Avance 500, and Bruker 600 MHz spectrometers using tetramethyl silane (TMS) as the internal standard. Chemical shifts are reported in parts per million (ppm) downfield from tetramethyl silane. Spin multiplicities are described as s (singlet), brs (broad singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), and/or m (multiplet). Coupling constants are reported in Hertz (Hz). Melting points were determined in a capillary tube using an Electrothermal apparatus (Model IA9200) and are uncorrected. The IR spectra were recorded by employing a Nicolet FTIR model MX-1 spectrophotometer. Analytical thin layer chromatography (TLC) was performed on MERCK precoated silica gel 60-F254 (0.5-mm) glass plates. Visualization of the spots on TLC plates was achieved either by exposure to iodine vapour or UV light or by dipping the plates into methanolic sulphuric acid-*β*-naphthol or to ethanolic anisaldehyde-sulphuric acid-acetic acid or to ethanolic ninhydrin solution and heating the plates to 120 °C. Column chromatography was performed using silica gel 60–120 and 100–200 mesh. Moisture sensitive reactions were carried out using standard syringe septum Techniques and under inert atmosphere of nitrogen. All solvents and reagents were purified by standard techniques. All evaporation of solvents was carried out under reduced pressure on Laborota-4000 rotary evaporator below 45 °C. The names of all the compounds given in the experimental section were taken from Chem Ultra, Version 11.0.

*2.1. General method for the synthesis of substituted nitro phenylcinnamides (**9a-b** and **15a-b**)*

To the ice cold solution of cinnamic acids (1 mmol) in dry dichloromethane added the oxalyl chloride (3 mmol) and a catalytic amount of N,N-dimethyl formamide (1 mol%) and stirred for 3 h at room temperature after completion of reaction and also excess of solvent and oxalyl chloride was removed under reduced pressure to give respected acid chlorides. These were dissolved in dry tetrahydrofuran and added to stock solutions of 3,4,5-trimethoxy anilines (0.9 mmol) and triethylamine (3 mmol) in dry tetrahydrofuran at 0 °C and stirred for 12 h at room temperature after completion of reaction and the reaction mixture was diluted with ethyl acetate, washed with water and brine solution, dried over sodium sulphate and purified by column chromatography using ethyl acetate and hexane as elutents.

*2.1.1. (E)-3-(4-Methoxy-3-nitrophenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**9a**)*

The compound **9a** was prepared according to the general method, employing (*E*)-3-(4-methoxy-3-nitrophenyl)acrylic acid **8a**, 500 mg, (2.24 mmol) and 3,4,5-trimethoxyaniline (450 mg, 2.46 mmol) to obtain the pure product **9a** as a pale yellow solid. Yield 69%; m.p: 198 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.04 (s, 1H), 7.71–7.62 (m, 2H), 7.56 (s, 1H), 7.11 (d, J = 9.0 Hz, 1H), 6.98 (s, 2H), 6.52 (d, J = 15.1 Hz, 1H), 4.00 (s, 3H), 3.86 (s, 6H), 3.83 (s, 3H); MS (ESI, m/z): 389 $[M+H]^+$.

*2.1.2. (E)-3-(3,4-Dimethoxy-5-nitrophenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**9b**)*

The compound **9b** was prepared according to the general method, employing (*E*)-3-(3,4-dimethoxy-5-nitrophenyl)acrylic acid **8b** 500 mg, (1.98 mmol) and 3,4,5-trimethoxyaniline (398 mg, 2.17 mmol) to obtain the pure product **9b** as a pale yellow solid. Yield 71%; m.p: 212 °C; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO) δ 9.63 (s, 1H), 7.65–7.54 (m, 2H), 7.46 (s, 1H), 7.09 (s, 2H), 6.81 (d, J = 15.6 Hz, 1H), 4.04–3.96 (m, 6H), 3.91–3.85 (m, 6H), 3.84–3.79 (m, 3H); MS (ESI, m/z): 419 $[M+H]^+$.

*2.1.3. (E)-3-(3-(Allyloxy)-4-nitrophenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**15a**)*

The compound **15a** was prepared according to the general method, employing (*E*)-3-(3-(allyloxy)-4-nitrophenyl)acrylic acid **14a**, 500 mg, (2.01 mmol) and 3,4,5-trimethoxyaniline (404 mg, 2.21 mmol) to obtain the pure product **15a** as a pale yellow solid. Yield 68%; m.p: 241 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.88 (d, J = 8.3 Hz, 1H), 7.70–7.65 (m, 2H), 7.17–7.14 (m, 2H), 6.97 (s, 2H), 6.58 (d, J = 15.1 Hz, 1H), 6.11–5.97 (m, 1H), 5.49 (dd, J = 1.51 Hz, J = 17.3 Hz, 1H), 5.37 (dd, J = 1.51 Hz, J = 10.5 Hz, 1H), 4.70 (d, J = 5.2 Hz, 2H), 3.86 (s, 6H), 3.84 (s, 3H); MS (ESI, m/z): 415 $[M+H]^+$.

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*2.1.4. (E)-3-(4-Nitro-3-(prop-2-ynyloxy)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**15b**)*

The compound **15b** was prepared according to the general method, employing (*E*)-3-(4-nitro-3-(prop-2-ynyloxy)phenyl)acrylic acid **14b**, 500 mg, (2.02 mmol) and 3,4,5-trimethoxyaniline (406 mg, 2.22 mmol) to obtain the pure product **15b** as a pale yellow solid. Yield 70%; m.p: 237 °C, ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO) δ (ppm): 10.00 (s, 1H), 7.89 (d, J = 8.3 Hz, 1H), 7.62 (d, J = 15.4 Hz, 1H), 7.47 (s, 1H), 7.31 (d, J = 8.3 Hz, 1H), 7.11 (s, 2H), 6.92 (d, J = 15.4 Hz, 1H), 4.96 (d, J = 2.2 Hz, 2H), 3.86 (s, 6H), 3.78 (s, 3H), 2.97 (t, J = 2.2 Hz, 1H); MS (ESI, m/z): 413 $[M+H]^+$.

*2.2. General method for the synthesis of substituted amino phenylcinnamides (**10a-b** and **16a-b**)*

Compounds **9a, 9b** and **15a, 15b** (1 mmol) were dissolved in methanol and added the zinc powder (3 mmol) and ammonium formate (3 mmol) portion wise at 0 °C and stirred at room temperature for 12 h after completion of reaction, the reaction mixture was filtered to remove the residual zinc, the solvent was removed under reduced pressure, the residue was dissolved in ethyl acetate and washed with water and brine solution and dried over sodium sulphate and solvent was removed under reduced pressure and purified by column chromatography using ethyl acetate and hexane as eluents.

*2.2.1. (E)-3-(3-Amino-4-methoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**10a**)*

The compound **10a** was prepared according to the general method, employing (*E*)-3-(4-methoxy-3-nitrophenyl)-N-(3,4,5-trimethoxyphenyl) acryl amide. 600 mg, (1.55 mmol) to obtain the pure product **10a** as a pale yellow solid. Yield 65%; m.p: 126 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.64 (d, J = 15.4 Hz, 1H), 7.44 (bs, 1H), 6.96 (s, 2H), 6.93–6.86 (m, 2H), 6.78–6.75 (m, 1H), 6.33 (d, J = 15.4 Hz, 1H), 3.88 (s, 3H), 3.86 (s, 6H), 3.82 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 164.7, 153.2, 149.0, 142.5, 136.4, 134.5, 134.4, 127.5, 119.9, 118.0, 112.9, 110.1, 97.5, 60.9, 55.9, 55.5; IR (KBr) (ν_{max}/cm^{-1}): 3380, 3004, 2935, 2839, 1681, 1606, 1546, 1507, 1452, 1429, 1411, 1343, 1301, 1263, 1235, 1210, 1186, 1168, 1128, 1018, 994, 972, 925, 886, 849, 836, 801; MS (ESI, m/z): 359 $[M+H]^+$; HRMS (ESI m/z) Calculated for $C_{19}H_{23}O_5N_2$: 359.1601, found: 359.1594 $[M+H]^+$.

*2.2.2. (E)-3-(3-Amino-4,5-dimethoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**10b**)*

The compound **10b** was prepared according to the general method, employing **(***E***)**-3-(3,4-dimethoxy-5-nitrophenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (600 mg, 1.44 mmol) to obtain the pure product **10b** pale yellow solid Yield 71%; m.p: 191 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.76 (bs, 1H), 7.55 (d, J = 15.4 Hz, 1H), 7.05 (s, 2H), 6.57–6.50 (m, 3H), 3.98 (bs, 2H), 3.85–3.80 (m, 15H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 164.4, 153.0, 152.7, 141.7, 140.7, 137.1, 134.8, 134.2, 130.6, 120.1, 108.1, 101.9, 97.4, 60.7, 59.8, 55.8, 55.5; IR (KBr) (ν_{max}/cm^{-1}): 3467, 3346, 3299, 2938, 1672, 1612, 1583, 1542, 1505, 1449, 1428, 1412, 1323, 1297, 1282, 1235, 1205, 1187, 1128, 998, 977; MS (ESI, m/z): 389 $[M+H]^+$; HRMS (ESI m/z) Calculated for $C_{20}H_{25}O_6N_2$: 389.1707, found: 389.1702 $[M+H]^+$.

*2.2.3. (E)-3-(3-(Allyloxy)-4-aminophenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**16a**)*

The compound **16a** was prepared according to the general method, (*E*)-3-(3-(allyloxy)-4-nitrophenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide**.** 600 mg, (1.45 mmol) to obtain the pure product **16a** white solid. Yield: 62%; m.p: 156 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.66 (d, J = 15.2 Hz, 1H), 7.50–7.41 (m, 1H), 7.30–7.20 (m, 1H), 6.96 (brs, 2H), 6.72–6.62 (m, 1H), 6.34 (d, J = 15.2 Hz, 1H), 6.19–5.98 (m, 1H), 5.48–5.20 (m, 2H), 4.63–4.45 (m, 2H), 4.18–4.01 (brs, 2H), 3.84 (s, 6H), 3.80 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 164.9, 153.2, 145.7, 142.8, 138.9, 134.6, 134.3, 132.9, 124.6, 122.5, 117.7, 116.2, 114.3, 111.0, 97.4, 69.1, 60.9, 55.9; IR (KBr) (ν_{max}/cm^{-1}): 3460, 3375, 3055, 2931, 2836, 1650, 1607, 1551, 1506, 1449, 1431, 1410, 1354, 1299, 1232, 1212, 1185, 1128, 1004, 983, 934, 849, 838, 819; MS (ESI, m/z): 385 $[M+H]^+$; HRMS (ESI, m/z) Calculated for $C_{21}H_{25}O_5N_2$: 385.1758, found: 385.1753 $[M+H]^+$.

*2.2.4. (E)-3-(4-Amino-3-(prop-2-ynyloxy)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**16b**)*

The compound **16b** was prepared according to the general method, (*E*)-3-(4-nitro-3-(prop-2-ynyloxy)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide. (600 mg, 1.46 mmol) to obtain the pure product **16b** brown solid Yield: 64%; m.p: 148 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.67 (d, J = 15.2 Hz, 1H), 7.37 (brs, 1H), 7.08 (s, 1H), 7.04 (d, J = 8.0 Hz, 1H), 6.95 (brs, 2H), 6.69 (d, J = 8.0 Hz, 1H), 6.33 (d, J = 15.2 Hz, 1H), 4.74 (d, J = 2.2 Hz, 2H), 4.14–4.04 (m, 1H), 3.85 (s, 6H), 3.82 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm: 164.8, 153.1, 144.7, 142.6, 139.1, 134.2, 124.6, 123.3, 116.4, 114.6, 111.5, 97.3, 78.2, 75.9, 60.9, 56.2, 55.9; IR (KBr) (ν_{max}/cm^{-1}): 3445, 3322, 3277, 2934, 2838, 2122, 1675, 1609, 1546, 1505, 1448, 1409, 1353, 1330, 1296, 1263, 1227, 1210, 1189, 1161, 1130, 1034, 1016, 995, 980, 922; MS (ESI, m/z): 383 $[M+H]^+$; HRMS (ESI, m/z) Calculated for $C_{21}H_{23}O_5N_2$: 383.1601, found: 383.1597 $[M+H]^+$.

*2.3. General procedure for the synthesis of 1-aryl-2-propyn-1-ol (**12**)*

A solution of aldehyde **11a-e** (5 mmol) in dry tetrahydrofuran was added to a stirred solution of ethynylmagnesium bromide in THF (0.5 M solution, 7.5 mmol) at 0 °C. The solution was stirred at 0 °C for 2 h and then warmed to room temperature and stirred for another 6–7 h. Saturated aqueous ammonium chloride solution 5 mL was added, and the mixture was evaporated in vacuo and partitioned between ethyl acetate and saturated ammonium chloride solution. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo to get pure compounds that were used for next step without further purification.

*2.4. General procedure of 1-arylprop-2-yn-1-one (**13**)*

To the stirred solution of 1-arylprop-2-yn-1-ol (1 mmol) in dimethyl sulfoxide (DMSO), a solution of 2-iodoxy-benzoic

acid (IBX) (1.1 mmol) in dimethyl sulfoxide (DMSO) (10 mL) was added at 10–15 °C. Then, the reaction mixture slowly raised the temperature to RT and allowed to stir for 4–6 h. The reaction was monitored by TLC using ethyl acetate/hexane (3:7) as a solvent system. Appropriate amount of water was added, the reaction mixture was filtered through Celite, and the aqueous layer was extracted with ethyl acetate. The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, and evaporated by using vacuum to get crude compounds. The compound was purified by column chromatography and the compound was eluted in ethyl acetate/hexane (3:7) as solvent system.

*2.4.1. 1-(3,4,5-Trimethoxyphenyl)prop-2-yn-1-one (**13a**)*

Compound **13a** was prepared according to the method described for compound **13**, employing 1-(3,4,5-trimethoxyphenyl) prop-2-yn-1-ol (**12a**, 750 mg, 3.38 mmol) and IBX (1.04 g, 3.72 mmol) to obtain the pure product **13a** as a light brown colour solid. (654 mg, 88% yield) m.p: 123–126 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.43 (s, 2H), 3.95 (s, 3H), 3.93 (s, 6H), 3.43 (s, 1H); MS (ESI) *m/z* 221 $[M+H]^+$.

*2.4.2. 1-(2-Bromo-3,4,5-trimethoxyphenyl) prop-2-yn-1-one (**13b**)*

Compound **13b** was prepared according to the method described for compound **13**, employing 1-(2-bromo-3,4,5-trime thoxyphenyl)prop-2-yn-1-ol (**12b**, 750 mg, 2.50 mmol) and IBX (770 mg, 2.75 mmol) to obtain the pure product **13b** as a brown colour solid. (638 mg, 86% yield); m.p: 80–81 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.46 (s, 1H), 3.98 (s, 3H), 3.93 (s, 3H), 3.89 (s, 3H), 3.50 (s, 1H); MS (ESI) *m/z* 298 $[M+H]^+$.

*2.4.3. 1-(3,4-Dimethoxy-5-nitrophenyl) prop-2-yn-1-one (**13c**)*

Compound **13c** was prepared according to the method described for compound **13**, employing 1-(3,4-dimethoxy-5-ni trophenyl)prop-2-yn-1-ol (**12c**, 750 mg, 3.16 mmol) and IBX (973 mg, 3.48 mmol) to obtain the pure product **13c** as a brown colour solid. (617 mg, 83% yield); m.p: 103–104 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.18 (s, 1H), 7.81 (s, 1H), 4.08 (s, 3H), 4.00 (s, 3H), 3.54 (s, 1H); MS (ESI) *m/z* 236 $[M+H]^+$.

*2.4.4. 1-(3-((tert-butyldimethylsilyl) oxy)-4-methoxyphenyl) prop-2-yn-1-one (**13d**)*

Compound **13d** was prepared according to the method described for compound **13**, employing 1-(3-((*tert*-butyldimethylsilyl) oxy)-4-methoxyphenyl) prop-2-yn-1-ol (**12d**, 750 mg, 2.57 mmol) and IBX (791 mg, 2.83 mmol) to obtain the pure product **13d** as a brown colour solid. (573 mg, 77% yield); m.p: 79–80 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 (dd, *J* = 8.5, 2.1 Hz, 1H), 7.62 (d, *J* = 2.1 Hz, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 3.90 (s, 3H), 3.36 (s, 1H), 1.01 (s, 9H), 0.21 (s, 6H); MS (EI) *m/z* 291 $[M+H]^+$.

*2.4.5. 1-(4-Methoxy-3-nitrophenyl)prop-2-yn-1-one (**13e**)*

Compound **13e** was prepared according to the method described for compound **13**, employing 1-(4-methoxy-3-nitro phenyl)prop-2-yn-1-ol (**12e**, 750 mg, 3.62 mmol) and IBX (1.11 g, 3.98 mmol) to obtain the pure product **13d** as a brown colour solid. (542 mg, 73% yield); m.p: 93–94 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm): 8.64 (d, *J* = 2.2 Hz, 1H), 8.34 (dd, *J* = 2.2 Hz, 9.06 Hz, 1H), 7.21 (d, *J* = 9.06 Hz, 1H), 4.08 (s, 3H), 3.53 (s, 1H); MS (EI) *m/z* 206 $[M+H]^+$.

*2.5. General procedure for the synthesis of target compounds **4–6 (a-h)** and **7a-f***

To the stirred solution of Aryl propynones **13(a-e)** (5 mmol) in absolute ethanol substituted amino phenylcinnamides **(10a-b and 16a-b)** (5 mmol) were added. The reaction was stirred for 3 h at room temperature. After the completion of reaction (checked by TLC), the reaction mixture was diluted with water and the crude product was filtered. The crude product was recrystallized from methanol to get pure yellow coloured compounds **4–6(a-h)** and **7a-f** with good yields.

*2.5.1. (E)-3-(4-Methoxy-3-((Z)-3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enylamino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**4a**)*

Yield (210 mg, 87%); yellow solid, m.p: 212 °C; ^{1}H NMR (300 MHz, DMSO) δ ppm: 12.14 (d, *J* = 12.4 Hz, 1H), 9.63 (s, 1H), 7.73–7.64 (dd, *J* = 8.0, 12.4 1H), 7.63–7.56 (m, 1H), 7.40 (s, 1H), 7.23 (s, 3H), 7.10 (s, 2H), 6.96 (d, *J* = 8.5 Hz, 1H), 6.67 (d, *J* = 15.6 Hz, 1H), 6.07 (d, *J* = 8.0 Hz, 1H), 4.00 (s, 3H), 3.91 (d, *J* = 4.0 Hz, 6H), 3.86 (s, 3H), 3.84 (s, 6H), 3.75 (d, *J* = 4.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$ + -DMSO) δ ppm: 188.79, 163.76, 152.32, 148.77, 142.86, 140.57, 139.51, 134.92, 133.97, 133.43, 129.38, 127.79, 122.80, 120.19, 111.83, 110.82, 104.26, 96.92, 93.70, 60.02, 55.59, 55.33; IR (KBr) (ν_{max}/cm^{-1}): 3369, 2895, 2841, 1619, 1548, 1524, 1489, 1410; MS (ESI): *m/z* 579 $[M+H]^+$; HRMS calcd for $C_{31}H_{35}O_9N_2$ $[M+H]^+$ 579.23371, found 579.23318.

*2.5.2. (E)-3-(3-((Z)-3-(2-Bromo-3,4,5-trimethoxyphenyl)-3-oxoprop-1-enylamino)-4-methoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**4b**)*

Yield (233 mg, 85%); yellow solid, m.p: 134 °C; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO) δ ppm: 11.96 (d, *J* = 12.8 Hz, 1H), 9.60 (bs, 1H), 7.69–7.54 (m, 2H), 7.44 (s, 1H), 7.28–7.24 (m, 1H), 7.11 (s, 2H), 6.98 (d, *J* = 8.5 Hz, 1H), 6.89 (s, 1H), 6.71 (d, *J* = 15.4 Hz, 1H), 5.77 (d, *J* = 7.7 Hz, 1H), 4.01 (s, 3H), 3.92 (s, 3H), 3.90 (s, 3H), 3.89 (s, 3H), 3.86 (s, 6H), 3.79 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 193.03, 164.27, 153.12, 152.82, 150.95, 149.56, 144.21, 143.57, 140.19, 138.06, 134.94, 134.24, 129.06, 127.79, 125.29, 120.29, 110.75, 110.70, 107.62, 106.06, 98.50, 97.17, 61.10, 61.05, 60.90, 56.14, 55.87, 55.83; IR (KBr) (ν_{max}/cm^{-1}): 3443, 2936, 2841, 1625, 1552, 1506, 1469, 1426; MS (ESI): *m/z* 657 $[M+H]^+$; HRMS calcd for $C_{31}H_{34}O_9N_2$ Br $[M+H]^+$ 657.14422, found 657.14464.

*2.5.3. (E)-3-(3-((Z)-3-(3,4-Dimethoxy-5-nitrophenyl)-3-oxoprop-1-enylamino)-4-methoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**4c**)*

Yield (216 mg, 85%); yellow solid, m.p: 165 °C; ^{1}H NMR (300 MHz, DMSO) δ ppm: 12.23 (d, *J* = 12.5 Hz, 1H), 9.79 (bs, 1H), 7.90 (s, 1H), 7.82 (s, 1H), 7.70–7.66 (m, 1H), 7.62

(d, J = 15.5 Hz, 1H), 7.53 (s, 1H), 7.29 (d, J = 7.8 Hz, 1H), 7.13 (s, 2H), 7.03 (d, J = 8.3 Hz, 1H), 6.73 (d, J = 15.5 Hz, 1H), 6.12 (d, J = 7.5 Hz, 1H), 4.04 (s, 9H), 3.87 (s, 6H), 3.78 (s, 3H); IR (KBr) (ν_{max}/cm^{-1}): 3440, 2941, 2840, 1682, 1648, 1608, 1535, 1508; MS (ESI): m/z 594 $[M+H]^+$; HRMS calcd for $C_{30}H_{32}O_{10}N_3$ $[M+H]^+$ 594.20822, found 594.20827.

*2.5.4. (E)-3-(3-((Z)-3-(3-Amino-4,5-dimethoxyphenyl)-3-oxoprop-1-enylamino)-4-methoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**4d**)*

Yield (179 mg, 76%); yellow solid, m.p: 127 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.13 (d, J = 12.4 Hz, 1H), 8.62 (bs, 1H), 7.62 (d, J = 15.4 Hz, 1H), 7.50–7.39 (dd, J = 7.9, 12.4, 1H), 7.22 (s, 1H), 7.10 (s, 2H), 7.03 (d, J = 9.8 Hz, 3H), 6.77 (d, J = 8.4 Hz, 1H), 6.57 (d, J = 15.4 Hz, 1H), 5.99 (d, J = 7.9 Hz, 1H), 3.87 (s, 6H), 3.83 (s, 6H), 3.82 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO) δ ppm: 189.65, 163.80, 155.63, 152.38, 151.98, 148.67, 142.18, 140.15, 139.44, 137.94, 134.79, 134.39, 129.67, 127.74, 122.78, 119.89, 111.44, 110.75, 107.63, 100.49, 96.94, 93.95, 60.08, 59.10, 55.51, 55.34, 55.13; IR (KBr) (ν_{max}/cm^{-1}): 3638, 3343, 3280, 1646, 1585, 1537, 1498; MS (ESI): m/z 564 $[M+H]^+$; HRMS calcd for $C_{30}H_{34}O_8N_3$ $[M+H]^+$ 564.23404, found 564.23319.

*2.5.5. (E)-3-(3-(((Z)-3-(3-((tert-butyldimethylsilyl)oxy)-4-methoxyphenyl)-3-oxoprop-1-en-1-yl)amino)-4-methoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**4e**)*

Yield (238 mg, 88%); yellow solid, m.p: 151 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.16 (d, J = 12.4 Hz, 1H), 8.88 (bs, 1H), 7.67–7.59 (m, 1H), 7.57–7.53 (m, 2H), 7.46 (dd, J = 12.4, 4.5 Hz, 1H), 7.20–7.15 (m, 3H), 6.99 (d, J = 8.4 Hz, 1H), 6.90 (d, J = 8.6 Hz, 1H), 6.74 (d, J = 8.4 Hz, 1H), 6.56 (d, J = 15.4 Hz, 1H), 6.09 (d, J = 7.9 Hz, 1H), 3.88 (s, 3H), 3.84 (s, 6H), 3.84 (s, 6H), 0.99 (s, 9H), 0.15 (s, 6H); MS (ESI): m/z 649 $[M+H]^+$.

*2.5.6. (E)-3-(3-((Z)-3-(3-Hydroxy-4-methoxyphenyl)-3-oxoprop-1-enylamino)-4-methoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**4f**)*

Yield (176 mg, 79%); yellow solid, m.p: 114 °C; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO) δ ppm: 12.07 (d, J = 12.4 Hz, 1H), 9.54 (bs, 1H), 8.19 (bs, 1H), 7.62 (d, J = 15.6 Hz, 1H), 7.58–7.47 (m, 3H), 7.36 (s, 1H), 7.21–7.16 (m, 1H), 7.13 (s, 2H), 6.95–6.88 (m, 2H), 6.69 (d, J = 15.6 Hz, 1H), 6.05 (d, J = 7.9 Hz, 1H), 3.98 (s, 3H), 3.93 (s, 3H), 3.87 (s, 6H), 3.80 (s, 3H); IR (KBr) (ν_{max}/cm^{-1}): 3314, 2933, 2840, 1665, 1608, 1547, 1508, 1479; MS (ESI): m/z 535 $[M+H]^+$; HRMS calcd for $C_{29}H_{31}O_8N_2$ $[M+H]^+$ 535.20749, found 535.20646.

*2.5.7. (E)-3-(4-Methoxy-3-((Z)-3-(4-methoxy-3-nitrophenyl)-3-oxoprop-1-enylamino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**4g**)*

Yield (202 mg, 86%); yellow solid, m.p: 133 °C; ^{1}H NMR (300 MHz, DMSO) δ ppm: 12.26 (d, J = 12.4 Hz, 1H), 9.78 (bs, 1H), 8.51 (s, 1H), 8.27 (d, J = 8.5 Hz, 1H), 7.80–7.70 (m, 1H), 7.65–7.58 (m, 2H), 7.48 (s, 1H), 7.29–7.21 (m, 2H), 7.12 (s, 1H), 6.99 (d, J = 8.3 Hz, 1H), 6.71 (d, J = 15.7 Hz, 1H), 6.09 (d, J = 7.3 Hz, 1H), 4.0 (s, 6H), 3.86 (s, 6H), 3.78 (s, 3H); IR (KBr) (ν_{max}/cm^{-1}): 3441, 2938, 2841, 1611, 1586, 1506, 1474, 1411; MS (ESI): m/z 564 $[M+H]^+$.

*2.5.8. (E)-3-(3-((Z)-3-(3-Amino-4-methoxyphenyl)-3-oxoprop-1-enylamino)-4-methoxyphenyl)-N-(3,5-dimethoxyphenyl)acrylamide (**4h**)*

Yield (174 mg, 78%); yellow solid, m.p: 144 °C; ^{1}H NMR (300 MHz, DMSO) δ ppm: 12.09 (d, J = 12.4 Hz, 1H), 9.67 (bs, 1H), 7.67–7.54 (m, 3H), 7.43–7.32 (m, 2H), 7.22 (d, J = 8.3 Hz, 1H), 7.13 (s, 2H), 6.97 (d, J = 8.4 Hz, 1H), 6.83 (d, J = 8.2 Hz, 1H), 6.71 (d, J = 15.5 Hz, 1H), 6.07 (d, J = 7.9 Hz, 1H), 4.18 (bs, 2H), 4.02 (s, 3H), 3.92 (s, 3H), 3.87 (s, 6H), 3.80 (d, J = 2.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO) δ ppm: 189.54, 163.77, 152.36, 149.40, 148.69, 141.79, 139.54, 136.20, 134.96, 133.39, 131.58, 129.72, 127.76, 122.60, 120.11, 117.37, 112.60, 111.29, 110.72, 108.85, 96.90, 93.96, 60.01, 55.53, 55.33, 54.94; IR (KBr) (ν_{max}/cm^{-1}): 3648, 3312, 3247, 2840, 1624, 1546, 1507, 1477; MS (ESI): m/z 534 $[M+H]^+$; HRMS calcd for $C_{29}H_{32}O_7N_3$ $[M+H]^+$ 534.22348, found 534.22216.

*2.5.9. (E)-3-(3,4-Dimethoxy-5-((Z)-3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enylamino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**5a**)*

Yield (190 mg, 81%); yellow solid, m.p: 208 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.20 (d, J = 12.8 Hz, 1H), 7.90 (s, 1H), 7.65 (d, J = 15.4 Hz, 1H), 7.54–7.42 (dd, J = 7.5, 12.8 Hz, 1H), 7.23 (s, 2H), 7.01 (s, 2H), 6.93 (s, 1H), 6.76 (s, 1H), 6.53 (d, J = 15.4 Hz, 1H), 6.04 (d, J = 7.5 Hz, 1H), 4.00 (s, 3H), 3.93 (s, 6H), 3.92 (s, 3H), 3.88 (s, 3H), 3.85 (s, 6H), 3.84 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO) δ ppm: 189.18, 163.53, 152.56, 152.44, 152.31, 142.60, 140.65, 139.73, 138.23, 134.60, 134.02, 133.76, 130.88, 121.45, 105.41, 104.28, 97.02, 93.84, 60.12, 55.59, 55.36; IR (KBr) (ν_{max}/cm^{-1}): 3389, 2980, 2841, 1626, 1565, 1508, 1467, 1426; MS (ESI): m/z 609 $[M+H]^+$; HRMS calcd for $C_{32}H_{37}O_{10}N_2$ $[M+H]^+$ 609.24427, found 609.24371.

*2.5.10. (E)-3-(3-((Z)-3-(2-Bromo-3,4,5-trimethoxyphenyl)-3-oxoprop-1-enylamino)-4,5-dimethoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**5b**)*

Yield (228 mg, 86%); yellow solid, m.p: 151 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.04 (d, J = 12.8 Hz, 1H), 7.96 (s, 1H), 7.64 (d, J = 15.3 Hz, 1H), 7.54–7.42 (dd, J = 7.5, 12.8 Hz, 1H), 6.99 (s, 2H), 6.96 (s, 1H), 6.89 (s, 1H), 6.76 (s, 1H), 6.56 (d, J = 15.4 Hz, 1H), 5.78 (d, J = 7.5 Hz, 1H), 3.99 (s, 3H), 3.91 (s, 3H), 3.90 (s, 3H), 3.89 (s, 3H), 3.87 (s, 3H), 3.83 (s, 6H), 3.82 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm: 192.92, 163.99, 153.24, 153.04, 152.83, 150.92, 144.29, 143.48, 141.12, 139.15, 138.07, 134.58, 133.85, 130.98, 121.36, 107.95, 107.35, 106.14, 105.17, 98.80, 97.50, 61.12, 61.05, 60.93, 60.90, 56.16, 55.99, 55.92; IR (KBr) (ν_{max}/cm^{-1}): 3445, 2985, 2840, 1646, 1589, 1535, 1489, 1468; MS (ESI): m/z 689 $[M+H]^+$; HRMS calcd for $C_{32}H_{38}O_{10}N_2$ Br $[M+H]^+$ 689.17043, found 689.15289.

*2.5.11. (E)-3-(3-((Z)-3-(3,4-Dimethoxy-5-nitrophenyl)-3-oxoprop-1-enylamino)-4,5-dimethoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**5c**)*

Yield (204 mg, 85%); yellow solid, m.p: 172 °C; ^{1}H NMR (300 MHz, DMSO) δ ppm: 12.29 (d, J = 12.8 Hz, 1H), 9.74 (s, 1H), 7.91–7.88 (m, 1H), 7.82–7.79 (m, 1H), 7.78–7.68 (dd, J = 7.7, 12.8 1H), 7.66–7.55 (m, 2H), 7.11 (s, 2H), 6.89 (s, 1H), 6.75 (d, J = 15.6 Hz, 1H), 6.09 (d, J = 7.7 Hz, 1H),

4.04 (s, 3H), 4.03 (s, 3H), 4.02 (s, 3H), 3.96 (s, 3H), 3.88 (s, 6H), 3.80 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO) δ ppm: 186.37, 163.50, 153.31, 152.52, 152.44, 144.41, 143.84, 143.66, 139.58, 138.27, 134.64, 134.02, 133.67, 133.34, 130.93, 121.61, 114.40, 113.92, 106.00, 105.35, 97.05, 93.01, 61.32, 60.18, 60.11, 55.97, 55.36; IR (KBr) (v_{max}/cm^{-1}): 3378, 2941, 2838, 1632, 1608, 1586, 1537; MS (ESI): *m/z* 624 $[M+H]^+$; HRMS calcd for $C_{31}H_{34}O_{11}N_3$ $[M+H]^+$ 624.21879, found 624.21885.

*2.5.12. (E)-3-(3-((Z)-3-(3-Amino-4,5-dimethoxyphenyl)-3-oxoprop-1-enylamino)-4,5-dimethoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**5d**)*

Yield (192 mg, 84%); yellow solid, m.p: 140 °C; ^{1}H NMR (300 MHz, DMSO) δ ppm: 12.18 (d, *J* = 12.1 Hz, 1H), 9.64 (s, 1H), 7.61 (d, *J* = 15.6 Hz, 1H), 7.58–7.54 (m, 2H), 7.11 (s, 2H), 7.04 (d, *J* = 7.3 Hz, 1H), 6.96 (s, 1H), 6.84 (s, 1H), 6.73 (d, *J* = 15.6 Hz, 1H), 6.03 (d, *J* = 7.5 Hz, 1H), 4.26 (bs, 2H), 4.00 (s, 3H), 3.94 (s, 3H), 3.92 (s, 3H), 3.87 (s, 6H), 3.86 (s, 3H), 3.80 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO) δ ppm: 189.85, 163.95, 163.57, 152.45, 152.41, 152.24, 152.00, 140.70, 140.48, 139.93, 139.80, 134.73, 134.60, 133.90, 130.26, 120.08, 107.78, 107.70, 101.02, 100.70, 97.06, 96.98, 60.13, 59.15, 55.34, 55.16, 55.04; IR (KBr) (v_{max}/cm^{-1}): 3450, 2933, 2849, 2360, 1625, 1548, 1506; MS (ESI): *m/z* 594 $[M+H]^+$; HRMS calcd for $C_{31}H_{36}O_9N_3$ $[M+H]^+$ 594.24461, found 594.24422.

*2.5.13. (E)-3-(3-((Z)-3-(3-Hydroxy-4-methoxyphenyl)-3-oxoprop-1-enylamino)-4,5-dimethoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**5f**)*

Yield (185 mg, 85%); yellow solid, m.p: 211 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 12.14 (d, *J* = 12.08 Hz, 1H), 8.31 (s, 1H), 7.64 (d, *J* = 15.8 Hz, 1H), 7.56–7.52 (m, 2H), 7.44–7.35 (dd, *J* = 8.3, 12.0 Hz, 1H), 7.04 (s, 2H), 6.90 (s, 2H), 6.70 (s, 1H), 6.59 (d, *J* = 15.8 Hz, 1H), 6.00 (d, *J* = 8.3 Hz, 1H), 3.97 (s, 3H), 3.95 (s, 3H), 3.85 (s, 12H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO) δ ppm: 189.39, 163.57, 152.54, 152.42, 150.17, 145.56, 141.88, 139.75, 134.67, 134.00, 133.60, 131.81, 130.86, 121.46, 119.35, 113.81, 109.97, 105.34, 105.02, 96.96, 93.98, 60.13, 60.08, 55.36, 55.32, 55.26; IR (KBr) (v_{max}/cm^{-1}): 3333, 2923, 2851, 2359, 1671, 1629, 1608, 1548; MS (ESI): *m/z* 565 $[M+H]^+$; HRMS calcd for $C_{30}H_{33}O_9N_2$ $[M+H]^+$ 565.21806, found 565.21720.

*2.5.14. (E)-3-(3,4-Dimethoxy-5-((Z)-3-(4-methoxy-3-nitrophenyl)-3-oxoprop-1-enylamino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**5g**)*

Yield (190 mg, 83%); yellow solid, m.p: 243 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.26 (d, *J* = 12.6 Hz, 1H), 9.52 (s, 1H), 8.02 (d, *J* = 15.6 Hz, 1H), 7.89–7.79 (m, 2H), 7.40 (s, 1H), 7.11 (s, 2H), 6.99 (s, 1H), 6.93 (d, *J* = 7.9 Hz, 1H), 6.77 (d, *J* = 15.6 Hz, 1H), 6.71–6.61 (m, 1H), 6.00 (d, *J* = 8.5 Hz, 1H), 4.06 (s, 3H), 4.03 (s, 3H), 3.99 (s, 3H), 3.96 (s, 3H), 3.84 (s, 6H); IR (KBr) (v_{max}/cm^{-1}): 3355, 3012, 1681, 1636, 1612, 1588, 1537; MS (ESI): *m/z* 594 $[M+H]^+$.

*2.5.15. (E)-3-(3-((Z)-3-(3-Amino-4-methoxyphenyl)-3-oxoprop-1-enylamino)-4,5-dimethoxyphenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**5h**)*

Yield (174 mg, 80%); yellow solid, m.p: 182 °C; ^{1}H NMR (300 MHz, DMSO) δ ppm: 12.16 (d, *J* = 12.4 Hz, 1H), 9.47 (bs, 1H), 7.61 (d, *J* = 15.4 Hz, 1H), 7.56–7.44 (m, 2H), 7.38 (s, 1H), 7.10 (s, 2H), 7.02 (s, 1H), 6.82 (d, *J* = 7.5 Hz, 1H), 6.71 (d, *J* = 15.7 Hz, 1H), 6.64–6.50 (m, 1H), 6.05 (d, *J* = 7.7 Hz, 1H), 4.00 (s, 3H), 3.92 (s, 3H), 3.87 (s, 6H), 3.82 (s, 6H); IR (KBr) (v_{max}/cm^{-1}): 3409, 2980, 2845, 2578, 1657, 1609, 1545, 1510; MS (ESI): *m/z* 564 $[M+H]^+$; HRMS calcd for $C_{30}H_{34}O_8N_3$ $[M+H]^+$ 564.23404, found 564.23314.

*2.5.16. (E)-3-(3-(Allyloxy)-4-((Z)-3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enylamino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**6a**)*

Yield (195 mg, 83%); yellow solid, m.p: 141 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.23 (d, *J* = 12.4 Hz, 1H), 7.77 (bs, 1H), 7.66 (d, *J* = 15.4 Hz, 1H), 7.57–7.46 (dd, *J* = 7.5, 12.4 Hz, 1H), 7.21 (s, 2H), 7.16–7.07 (m, 2H), 7.05–6.96 (m, 3H), 6.47 (d, *J* = 15.4 Hz, 1H), 6.21–6.10 (m, 1H), 6.06 (d, *J* = 7.5 Hz, 1H), 5.59 (d, *J* = 17.5 Hz, 1H), 5.36 (d, *J* = 10.6 Hz, 1H), 4.69 (d, *J* = 4.7 Hz, 2H), 3.92 (s, 6H), 3.91 (s, 3H), 3.84 (s, 6H), 3.83 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 190.08, 164.31, 153.27, 152.99, 147.17, 142.30, 141.41, 134.61, 132.27, 131.60, 129.80, 121.79, 119.59, 118.06, 112.96, 111.53, 104.89, 97.61, 95.13, 69.47, 60.91, 56.18, 55.98; IR (KBr) (v_{max}/cm^{-1}): 3321, 2927, 2840, 1645, 1608, 1525, 1489; MS (ESI): *m/z* 605 $[M+H]^+$; HRMS calcd for $C_{33}H_{37}O_9N_2$ $[M+H]^+$ 605.24936, found 605.24869.

*2.5.17. (E)-3-(3-(Allyloxy)-4-((Z)-3-(2-bromo-3,4,5-trimethoxyphenyl)-3-oxoprop-1-enylamino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**6b**)*

Yield (218 mg, 82%); yellow solid, m.p: 113 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.08 (d, *J* = 12.0 Hz, 1H), 7.67 (d, *J* = 15.1 Hz, 1H), 7.60 (bs, 1H), 7.55–7.46 (dd, *J* = 8.3, 12.0 Hz, 1H), 7.15 (s, 2H), 7.04 (s, 1H), 6.97 (s, 2H), 6.87 (s, 1H), 6.47 (d, *J* = 15.4 Hz, 1H), 6.22–6.05 (m, 1H), 5.80 (d, *J* = 8.3 Hz, 1H), 5.64 (d, *J* = 17.2 Hz, 1H), 5.37 (d, *J* = 10.6 Hz, 1H), 4.69 (d, *J* = 3.7 Hz, 2H), 3.91 (s, 3H), 3.90 (s, 3H), 3.86 (s, 3H), 3.85 (s, 6H), 3.83 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 192.82, 164.24, 153.17, 152.76, 150.87, 147.12, 144.17, 142.20, 141.27, 138.13, 134.64, 134.33, 132.01, 131.10, 130.11, 121.60, 119.83, 118.06, 113.01, 111.35, 107.87, 106.10, 99.40, 97.32, 69.29, 61.10, 61.01, 60.93, 56.09, 55.91; IR (KBr) (v_{max}/cm^{-1}): 3343, 2928, 2851, 1656, 1634, 1543, 1528; MS (ESI): *m/z* 683 $[M+H]^+$; HRMS calcd for $C_{33}H_{36}O_9N_2Br$ $[M+H]^+$ 683.15987, found 683.15987.

*2.5.18. (E)-3-(3-(Allyloxy)-4-((Z)-3-(3,4-dimethoxy-5-nitrophenyl)-3-oxoprop-1-enylamino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**6c**)*

Yield (207 mg, 86%); yellow solid, m.p: 134 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.28 (d, *J* = 12.8 Hz, 1H), 7.88 (d, *J* = 1.8 Hz, 1H), 7.78 (d, *J* = 1.8 Hz, 1H), 7.68 (d, *J* = 15.4 Hz, 1H), 7.63–7.54 (dd, *J* = 7.5, 12.8 Hz, 1H), 7.40 (s, 1H), 7.22–7.15 (m, 2H), 7.08 (s, 1H), 6.96 (s, 2H), 6.44 (d, *J* = 15.4 Hz, 1H), 6.26–6.09 (m, 1H), 6.04 (d, *J* = 7.5 Hz, 1H), 5.64 (d, *J* = 17.3 Hz, 1H), 5.41 (d, *J* = 10.6 Hz, 1H), 4.75 (d, *J* = 4.8 Hz, 2H), 4.04 (s, 3H), 4.00 (s, 3H), 3.87 (s, 6H), 3.84 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 187.09, 164.23, 153.96, 153.17, 147.10, 145.26, 144.06, 143.27, 141.35, 134.56, 134.41, 134.34, 132.12, 131.01, 130.11, 121.56, 119.68, 117.97, 115.18, 114.34, 113.13, 111.58, 97.38, 94.17, 69.33, 62.01, 60.91, 56.42, 55.90; IR (KBr) (v_{max}/cm^{-1}):

3421, 2937, 1628, 1600, 1536, 1506, 1473; MS (ESI): *m/z* 620 $[M+H]^+$; HRMS calcd for $C_{32}H_{34}O_{10}N_3$ $[M+H]^+$ 620.22387, found 620.22314.

*2.5.19. (E)-3-(3-(Allyloxy)-4-((Z)-3-(3-amino-4,5-dimethoxyphenyl)-3-oxoprop-1-enylamino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**6d**)*

Yield (195 mg, 85%); yellow solid, m.p: 212 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.27 (d, *J* = 12.8 Hz, 1H), 7.86 (d, *J* = 1.5 Hz, 1H), 7.77 (d, *J* = 1.51 Hz, 1H), 7.67 (d, *J* = 15.4 Hz, 1H), 7.62–7.52 (dd, *J* = 7.5, 12.8 Hz, 2H), 7.22–7.11 (m, 2H), 7.05 (s, 1H), 6.98 (s, 2H), 6.46 (d, *J* = 15.4 Hz, 1H), 6.22–6.07 (m, 1H), 6.03 (d, *J* = 7.5 Hz, 1H), 5.63 (d, *J* = 17.0 Hz, 1H), 5.40 (d, *J* = 10.5 Hz, 1H), 4.72 (d, *J* = 4.8 Hz, 2H), 4.03 (s, 3H), 3.99 (s, 3H), 3.85 (s, 6H), 3.84 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 187.14, 164.16, 154.00, 153.22, 147.15, 145.31, 144.11, 143.27, 141.44, 134.45, 132.15, 131.08, 130.11, 121.63, 119.63, 118.01, 115.23, 114.36, 113.16, 111.60, 97.39, 94.21, 69.38, 62.04, 60.93, 56.45, 55.94; IR (KBr) (ν_{max}/cm^{-1}): 3317, 2923, 2850, 1678, 1625, 1538, 1507; MS (ESI): *m/z* 590 $[M+H]^+$; HRMS calcd for $C_{32}H_{36}O_8N_3$ $[M+H]^+$ 590.24969, found 590.24933.

*2.5.20. (E)-3-(3-(allyloxy)-4-(((Z)-3-(3-((tert-butyldimethylsilyl)oxy)-4-methoxyphenyl)-3-oxoprop-1-en-1-yl)amino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**6e**)*

Yield (226 mg, 86%); yellow solid, m.p: 183 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 12.22 (d, *J* = 12.4 Hz, 1H), 7.66 (d, *J* = 15.2 Hz, 1H), 7.59–7.55 (m, 2H), 7.51–7.44 (m, 2H), 7.12 (s, 2H), 7.03 (s, 1H), 6.98 (s, 1H), 6.87 (d, *J* = 8.5 Hz, 1H), 6.43 (d, *J* = 15.2 Hz, 1H), 6.16–6.08 (m, 1H), 6.05 (d, *J* = 8.0 Hz, 1H), 5.63 (d, *J* = 17.9 Hz, 1H), 5.37 (d, *J* = 10.6 Hz, 1H), 4.70 (d, *J* = 4.7 Hz, 2H), 3.86 (s, 9H), 3.83 (s, 3H), 1.60 (s, 9H), 1.01 (s, 6H); MS (ESI): *m/z* 675 $[M+H]^+$.

*2.5.21. (E)-3-(3-(Allyloxy)-4-((Z)-3-(3-hydroxy-4-methoxyphenyl)-3-oxoprop-1-enylamino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**6f**)*

Yield (172 mg, 79%); yellow solid, m.p: 196 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.22 (d, *J* = 12.0 Hz, 1H), 7.65 (d, *J* = 15.8 Hz, 1H), 7.58–7.50 (m, 2H), 7.50–7.41 (dd, *J* = 7.5, 12.0 Hz, 1H), 7.09 (s, 2H), 6.99 (d, *J* = 6.9 Hz, 3H), 6.87 (d, *J* = 8.3 Hz, 1H), 6.43 (d, *J* = 15.3 Hz, 1H), 6.19–6.08 (m, 1H), 6.04 (d, *J* = 7.5 Hz, 1H), 5.73 (bs, 1H), 5.62 (d, *J* = 17.3 Hz, 1H), 5.36 (d, *J* = 10.7 Hz, 1H), 4.66 (d, *J* = 2.0 Hz, 2H), 3.94 (s, 3H), 3.85 (s, 6H), 3.83 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 190.02, 153.23, 149.68, 147.03, 145.33, 141.80, 141.69, 134.48, 132.66, 132.24, 131.77, 130.88, 129.39, 128.76, 121.88, 120.56, 119.21, 117.90, 113.70, 112.67, 111.40, 109.95, 97.40, 95.19, 69.32, 68.13, 60.97, 56.00; IR (KBr) (ν_{max}/cm^{-1}): 3394, 2924, 2852, 2359, 1624, 1594, 1548, 1506; MS (ESI): *m/z* 561 $[M+H]^+$.

*2.5.22. (E)-3-(3-(Allyloxy)-4-((Z)-3-(4-methoxy-3-nitrophenyl)-3-oxoprop-1-enylamino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**6g**)*

Yield (190 mg, 83%); yellow solid, m.p: 201 °C; ^{1}H NMR (300 MHz, DMSO) δ ppm: 12.31 (d, *J* = 12.5 Hz, 1H), 9.49 (s, 1H), 8.45 (s, 1H), 8.19 (d, *J* = 8.7 Hz, 1H), 7.68–7.58 (m, 2H), 7.49 (s, 1H), 7.27–7.17 (m, 3H), 7.12 (s, 2H), 7.11 (s, 1H), 6.66 (d, *J* = 15.6 Hz, 1H), 6.08 (d, *J* = 7.8 Hz, 1H), 5.71 (d, *J* = 17.3 Hz, 1H), 5.43 (d, *J* = 10.7 Hz, 1H), 4.77 (s, 2H), 4.05 (s, 3H), 3.88 (s, 6H), 3.81 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ ppm: 186.23, 163.74, 154.34, 152.38, 146.49, 142.82, 139.56, 138.68, 134.87, 132.58, 131.80, 130.90, 130.43, 129.89, 124.13, 121.33, 120.11, 117.21, 112.79, 112.72, 110.82, 96.83, 93.48, 68.74, 60.11, 56.25, 55.34; IR (KBr) (ν_{max}/cm^{-1}): 3433, 2828, 1621, 1594, 1548, 1509, 1498; MS (ESI): *m/z* 590 $[M+H]^+$; HRMS calcd for $C_{31}H_{32}O_9N_3$ $[M+H]^+$ 590.21331, found 590.21301.

*2.5.23. (E)-3-(3-(Allyloxy)-4-((Z)-3-(3-amino-4-methoxyphenyl)-3-oxoprop-1-enylamino)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**6h**)*

Yield (183 mg, 84%); yellow solid, m.p: 215 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.27 (m, 1H), 8.16–8.07 (m, 1H), 7.93–7.82 (m, 1H), 7.64 (d, *J* = 15.2 Hz, 1H), 7.37 (d, *J* = 8.7 Hz, 1H), 7.12–6.93 (m, 6H), 6.67 (d, *J* = 8.0 Hz, 1H), 6.46 (dd, *J* = 15.3, 7.3 Hz, 1H), 6.34 (d, *J* = 15.3 Hz, 1H), 6.19–5.96 (m, 2H), 5.62 (d, *J* = 17.5 Hz, 1H), 5.45–5.27 (m, 1H), 4.72–4.54 (m, 2H), 3.99 (s, 3H), 3.84 (s, 6H), 3.82 (s, 3H); IR (KBr) (ν_{max}/cm^{-1}): 3373, 2924, 2852, 1592, 1545, 1506, 1474; MS (ESI): *m/z* 560 $[M+H]^+$; HRMS calcd for $C_{31}H_{34}O_7N_3$ $[M+H]^{+v}$ 560.23913, found 560.23850.

*2.5.24. (E)-3-(4-((Z)-3-Oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enylamino)-3-(prop-2-ynyloxy)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**7a**)*

Yield (186 mg, 79%); yellow solid, m.p: 220 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.19 (d, *J* = 12.5 Hz, 1H), 7.71 (d, *J* = 15.3 Hz, 1H), 7.65 (s, 1H), 7.52 (dd, *J* = 12.4, 8.1 Hz, 1H), 7.26 (s, 1H), 7.22 (s, 2H), 7.17 (s, 2H), 6.98 (s, 2H), 6.48 (d, *J* = 15.3 Hz, 1H), 6.08 (d, *J* = 8.0 Hz, 1H), 4.90 (s, 2H), 3.93 (s, 6H), 3.92 (s, 3H), 3.86 (s, 6H), 3.84 (s, 3H), 2.58 (s, 1H); IR (KBr) (ν_{max}/cm^{-1}): 3356, 3268, 2841, 1668, 1632, 1570, 1548; MS (ESI): *m/z* 603 $[M+H]^+$; HRMS calcd for $C_{33}H_{35}O_9N_2$ $[M+H]^+$ 603.23371, found 603.23352.

*2.5.25. (E)-3-(4-((Z)-3-(2-Bromo-3,4,5-trimethoxyphenyl)-3-oxoprop-1-enylamino)-3-(prop-2-ynyloxy)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**7b**)*

Yield (229 mg, 86%); yellow solid, m.p: 187 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.02 (d, *J* = 12.7 Hz, 1H), 7.70 (d, *J* = 15.2 Hz, 2H), 7.53–7.45 (m, 1H), 7.23 (s, 1H), 7.16 (s, 2H), 6.98 (s, 2H), 6.88 (s, 1H), 6.50 (d, *J* = 15.4 Hz, 1H), 5.82 (d, *J* = 7.7 Hz, 1H), 4.87 (s, 2H), 3.92 (s, 3H), 3.90 (s, 3H), 3.86 (s, 3H), 3.85 (s, 6H), 3.83 (s, 3H), 2.58 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 192.84, 164.21, 153.18, 152.74, 150.89, 146.05, 144.27, 142.36, 141.17, 137.94, 134.65, 134.31, 131.27, 130.09, 122.36, 120.05, 113.35, 111.76, 107.95, 106.20, 99.52, 97.33, 77.52, 76.83, 61.11, 61.02, 60.94, 56.38, 56.10, 55.91; IR (KBr) (ν_{max}/cm^{-1}): 3447, 2935, 2851, 1674, 1648, 1575, 1555; MS (ESI): *m/z* 681 $[M+H]^+$; HRMS calcd for $C_{33}H_{34}O_9N_2$ Br $[M+H]^+$ 681.14422, found 681.14415.

*2.5.26. (E)-3-(4-((Z)-3-(3,4-Dimethoxy-5-nitrophenyl)-3-oxoprop-1-enylamino)-3-(prop-2-ynyloxy)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**7c**)*

Yield (193 mg, 80%); yellow solid, m.p: 216 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.30 (d, *J* = 12.6 Hz, 1H), 8.90 (s, 1H), 7.79–7.68 (m, 3H), 7.62 (s, 1H), 7.43 (s, 1H), 7.31 (d, *J* = 7.4 Hz, 1H), 7.20 (s, 2H), 6.69 (d, *J* = 7.4 Hz, 1H), 6.58

(d, J = 15.6 Hz, 1H), 6.13 (d, J = 8.0 Hz, 1H), 4.96 (d, J = 6.4 Hz, 1H), 4.81 (d, J = 2.2 Hz, 1H), 4.06 (s, 3H), 4.05 (s, 3H), 3.99 (s, 6H), 3.86 (s, 3H), 2.59 (s, 1H); IR (KBr) (v_{max}/cm^{-1}): 3366, 3277, 2925, 1674, 1636, 1598, 1573; MS (ESI): m/z 618 $[M+H]^+$; HRMS calcd for $C_{32}H_{32}O_{10}N_3$ $[M+H]^+$ 618.20822, found 618.20835.

*2.5.27. (E)-3-(4-((Z)-3-(3-Amino-4,5-dimethoxyphenyl)-3-oxoprop-1-enylamino)-3-(prop-2-ynyloxy)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**7d**)*

Yield (174 mg, 77%); yellow solid, m.p: 118 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.19 (m, 1H), 7.73–7.63 (m, 2H), 7.41 (s, 1H), 7.23 (s, 1H), 7.14 (s, 1H), 7.07 (d, J = 6.3 Hz, 1H), 7.05–6.96 (m, 4H), 6.69 (d, J = 8.0 Hz, 1H), 6.51–6.42 (m, 1H), 6.34 (d, J = 15.3 Hz, 1H), 6.08–5.98 (m, 1H), 4.89 (d, J = 6.5 Hz, 1H), 4.74 (d, J = 2.2 Hz, 1H), 3.90 (s, 3H), 3.87 (s, 3H), 3.85 (s, 6H), 3.83 (s, 3H), 2.56 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 190.65, 164.36, 153.17, 152.69, 145.85, 142.13, 141.28, 140.27, 138.79, 134.89, 134.71, 134.31, 131.64, 129.52, 122.47, 113.02, 111.77, 111.50, 108.34, 101.53, 97.41, 95.34, 77.66, 76.76, 60.93, 59.92, 56.31, 55.91, 55.71; IR (KBr) (v_{max}/cm^{-1}): 3261, 2924, 2851, 1625, 1593, 1544, 1506. MS (ESI): m/z 588 $[M+H]^+$; HRMS calcd for $C_{32}H_{34}O_8N_3$ $[M+H]^+$ 588.23404, found 588.23373.

*2.5.28. (E)-3-(4-(((Z)-3-(3-((tert-butyldimethylsilyl)oxy)-4-methoxyphenyl)-3-oxoprop-1-en-1-yl)amino)-3-(prop-2-yn-1-yloxy)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**7e**)*

Yield (229 mg, 87%); yellow solid, m.p: 167 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 12.15 (d, J = 12.2 Hz, 1H), 7.70 (d, J = 15.4 Hz, 1H), 7.57 (dd, J = 8.3, 1.9 Hz, 1H), 7.51 (d, J = 1.9 Hz, 1H), 7.46 (dd, J = 12.2, 8.0 Hz, 1H), 7.24 (s, 1H), 7.13 (s, 2H), 7.00 (s, 2H), 6.87 (d, J = 8.5 Hz, 1H), 6.48 (d, J = 15.4 Hz, 1H), 6.06 (d, J = 8.0 Hz, 1H), 4.87 (s, 2H), 3.86 (s, 3H), 3.85 (s, 6H), 3.84 (s, 3H), 2.55 (s, 1H), 1.01 (s, 9H), 0.18 (s, 6H); MS (ESI): m/z 673 $[M+H]^+$.

*2.5.29. (E)-3-(4-((Z)-3-(3-Hydroxy-4-methoxyphenyl)-3-oxoprop-1-enylamino)-3-(prop-2-ynyloxy)phenyl)-N-(3,4,5-trimethoxyphenyl)acrylamide (**7f**)*

Yield (181 mg, 83%); yellow solid, m.p: 170 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 9.70 (bs, 1H), 8.57 (bs, 1H), 7.63 (d, J = 16.0 Hz, 1H), 7.58–7.54 (m, 2H), 7.51 (s, 1H), 7.32 (s, 1H), 7.24 (s, 2H), 7.12 (s, 2H), 6.91 (d, J = 8.4 Hz, 1H), 6.69 (d, J = 15.6 Hz, 1H), 6.09 (d, J = 8.0 Hz, 1H), 4.97 (s, 2H), 3.94 (s, 3H), 3.87 (s, 6H), 3.79 (s, 3H), 2.59 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO) δ ppm: 189.34, 163.94, 152.42, 150.40, 145.66, 145.28, 141.29, 139.80, 134.93, 133.36, 131.62, 131.20, 129.07, 122.22, 119.82, 119.50, 113.86, 112.56, 111.08, 110.03, 96.81, 94.63, 77.49, 60.21, 55.88, 55.35, 55.30; IR (KBr) (v_{max}/cm^{-1}): 3360, 2926, 1630, 1598, 1546, 1507; MS (ESI): m/z 559 $[M+H]^+$.

3. Biology

3.1. MTT assay

The cytotoxic activity of the compounds was determined using MTT assay (Botta et al., 2007) Cells were seeded in 200 µL DMEM, supplemented with 10% FBS in each well of 96-well microculture plates and incubated for 24 h at 37 °C in a CO_2 incubator. After 24 h of incubation cells were treated with test compounds 48 h. After 48 h of incubation, 10 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/ml) was added to each well and the plates were further incubated for 4 h. Then the supernatant from each well was carefully removed, formazan crystals were dissolved in 200 µL of DMSO and absorbance at 570 nm wavelength was recorded.

3.2. Cell cycle analysis

Flow cytometric analysis (FACS) was performed to evaluate the distribution of the cells through the cell-cycle phases. DU-145 cells, prostate cancer cells were incubated for 48 h with compounds **6d** and **6g** at concentrations of 5 and 10 µM. Untreated and treated cells were harvested, washed with phosphate-buffered saline (PBS), fixed in ice-cold 70% ethanol, and stained with propidium iodide (Sigma–Aldrich). Cell-cycle analysis was performed by flow cytometry (Becton Dickinson FACS Calibur instrument) (Szumilak et al., 2010).

3.3. Tubulin polymerization assay

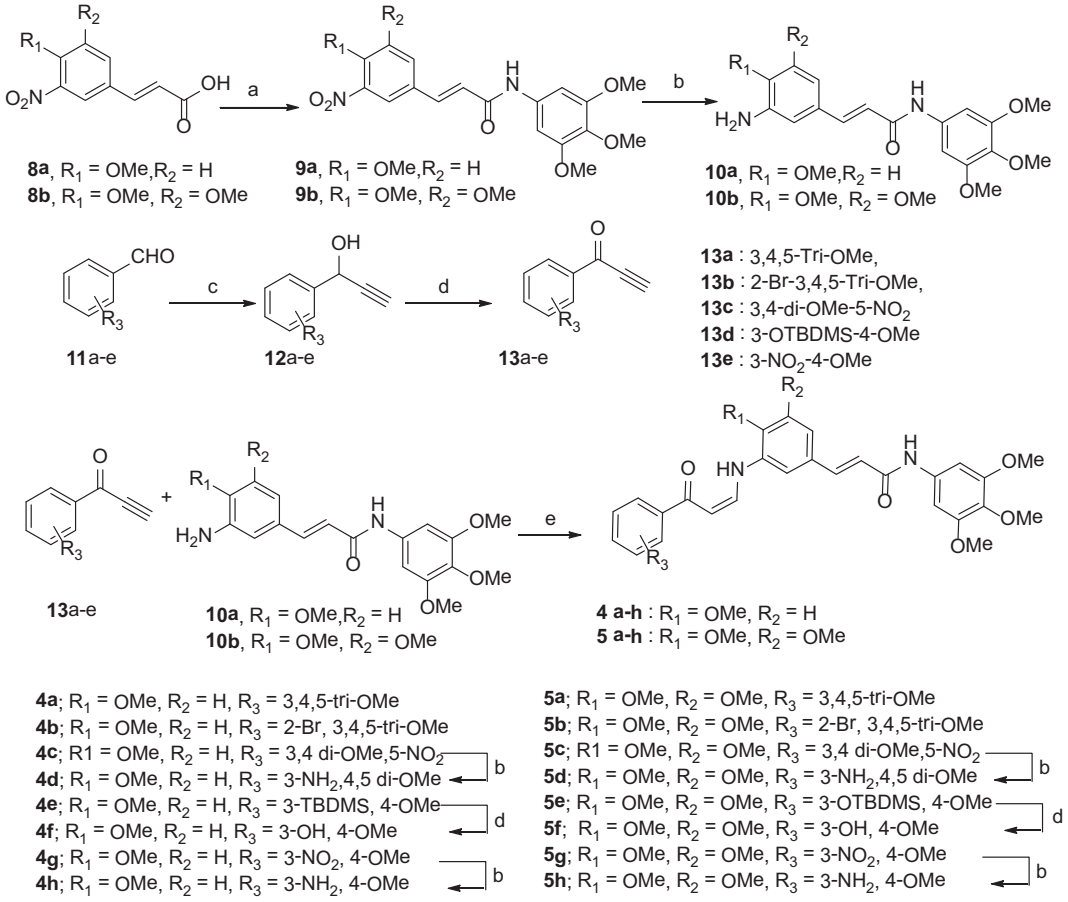
A fluorescence based *in vitro* tubulin polymerization assay was performed according to the manufacturer's protocol (BK011, Cytoskeleton, Inc.). Briefly, the reaction mixture in a total volume of 10 µL contained PEM buffer, GTP (1 µM) in the presence or absence of test compounds. Tubulin polymerization was followed by a time dependent increase in fluorescence due to the incorporation of a fluorescence reporter into microtubules as polymerization proceeds. Fluorescence emission at 420 nm (excitation wavelength is 360 nm) was measured by using a Varioskan multimode plate reader (Thermo scientific Inc.). Cinnamide was used as reference compound in this study. To determine the IC50 values of the compounds against tubulin polymerization, the compounds were pre-incubated with tubulin at varying concentrations. Assay was performed under similar conditions as employed for polymerization assays as described above (Huber et al., 2008; Kamal et al., 2011).

3.4. Mitochondrial membrane potential

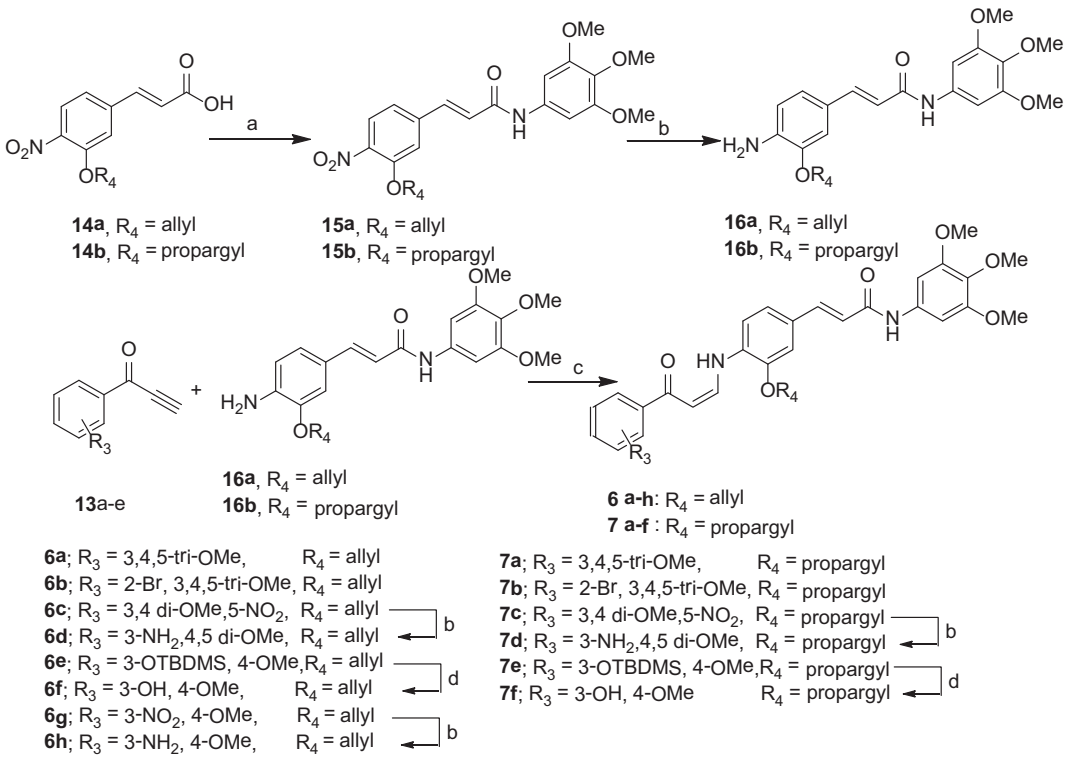
DU-145 (1×10^6 cells/well) cells were cultured in six-well plates after treatment with compounds **6d** and **6g** at 5 and 10 µM concentrations for 48 h. After 48 h of treatment, cells were collected by trypsinization and washed with PBS followed by resuspending in JC-1 (5 µg/ml) and incubated at 37 °C for 15 min. Cells were rinsed three times with medium and suspended in pre warmed medium. The cells were then subjected to flow cytometric analysis on a flow cytometer (Becton Dickinson) in the FL1, FL2 channel to detect mitochondrial potential (Chakravarti et al., 2012).

3.5. Annexin staining assay for apoptosis

DU-145 (1×10^6) cells were seeded in six-well plates and allowed to grow overnight. The medium was then replaced with complete medium containing compounds **6d** and **6g** at 5



Scheme 1



Scheme 2 Reagents and conditions: (a) (i) $(COCl)_2$, dry DCM, 0 °C to rt, 3 h; (ii) Trimethoxy aniline, Et_3N, dry THF, 3 h; (b) Zn, HCO_2NH_4, MeOH, rt, 6 h; (c) EtOH, 3–4 h; (d) TBAF, dry DCM, 0 °C to rt, 3 h; (e) EtOH, 3 h.

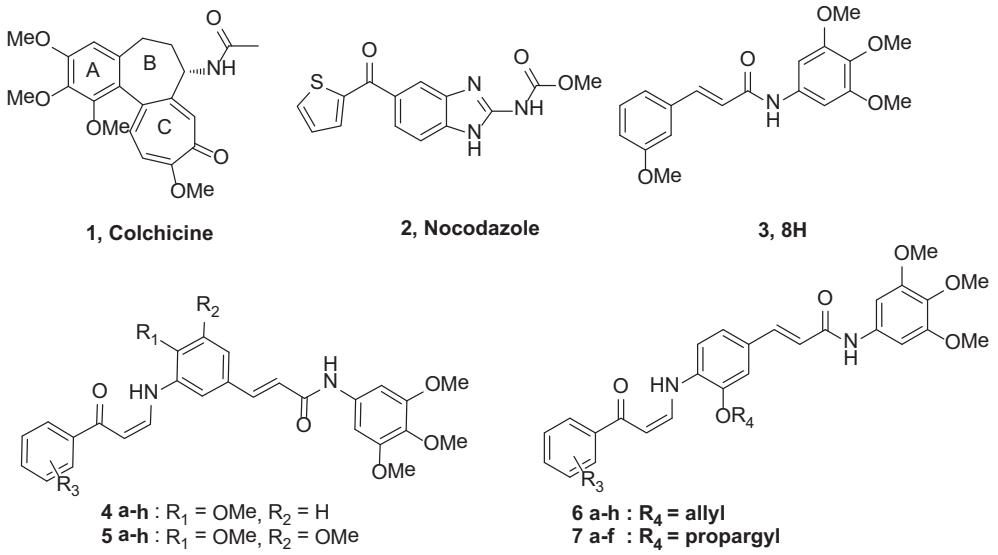


Figure 1 Tubulin polymerization inhibitors.

Table 1 Cytotoxicity (IC_{50} values in μM) of cinnamido-propionone conjugates (**4, 5, 6a-h** and **7a-f**) against a panel of human cancer cells.[a]

Compound	MCF-7[b]	A549[c]	DU-145[d]	HeLa[e]
4a	10.47 ± 0.91	19.38 ± 0.47	12.88 ± 0.80	9.772 ± 0.59
4b	32.92 ± 1.45	34.29 ± 1.74	12.58 ± 0.78	13.08 ± 0.43
4c	23.88 ± 1.17	28.33 ± 1.21	16.98 ± 0.86	18.75 ± 0.14
4d	14.13 ± 0.51	19.49 ± 0.64	15.84 ± 0.64	17.78 ± 0.91
4f	15.06 ± 0.45	12.84 ± 0.57	9.036 ± 0.58	12.51 ± 1.41
4g	18.46 ± 0.91	21.29 ± 1.13	11.48 ± 0.46	14.27 ± 1.08
4h	14.79 ± 0.32	8.912 ± 0.47	11.74 ± 0.17	13.59 ± 0.98
5a	79.43 ± 7.12	158.4 ± 7.47	45.18 ± 2.85	55.50 ± 3.02
5b	66.06 ± 3.93	77.83 ± 4.66	40.90 ± 2.37	68.71 ± 6.58
5c	70.79 ± 3.58	68.71 ± 4.52	46.00 ± 2.34	61.11 ± 3.54
5d	48.97 ± 2.74	93.32 ± 6.99	40.41 ± 2.69	59.13 ± 2.90
5f	17.51 ± 0.31	21.37 ± 1.02	9.141 ± 0.54	9.931 ± 0.16
5g	14.93 ± 0.91	19.95 ± 1.13	18.55 ± 0.94	9.935 ± 0.88
5h	22.85 ± 1.88	24.50 ± 1.27	12.58 ± 0.41	13.87 ± 0.26
6a	10.71 ± 0.64	22.00 ± 0.66	19.18 ± 1.21	20.07 ± 1.70
6b	125.8 ± 6.12	106.2 ± 5.03	42.20 ± 2.18	44.36 ± 2.38
6c	49.27 ± 2.30	39.50 ± 2.42	17.63 ± 1.31	29.83 ± 1.10
6d	11.48 ± 0.42	12.24 ± 0.61	7.481 ± 0.12	8.128 ± 0.19
6f	14.12 ± 0.77	16.36 ± 0.86	9.332 ± 0.30	13.94 ± 0.75
6g	14.71 ± 0.61	16.14 ± 0.81	8.912 ± 0.15	9.332 ± 0.49
6h	51.87 ± 2.05	58.44 ± 2.96	27.58 ± 1.09	38.28 ± 1.47
7a	16.31 ± 0.91	17.48 ± 0.39	18.72 ± 0.94	5.623 ± 0.28
7b	37.15 ± 0.24	35.77 ± 0.26	10.30 ± 0.74	17.38 ± 0.78
7c	42.75 ± 2.65	63.53 ± 3.20	21.38 ± 0.64	31.17 ± 2.24
7d	158.4 ± 0.65	87.16 ± 1.10	50.11 ± 2.60	93.32 ± 0.02
7f	11.74 ± 0.79	15.51 ± 0.50	24.80 ± 1.23	3.380 ± 0.17
8H	13.48 ± 0.54	9.772 ± 0.49	14.93 ± 0.57	16.93 ± 0.63

[a] 50% Growth inhibitory concentration and the values are average of three individual experiments after 48 h of drug treatment.
[b] Breast cancer.
[c] Lung cancer.
[d] Prostate cancer.
[e] Cervical cancer.

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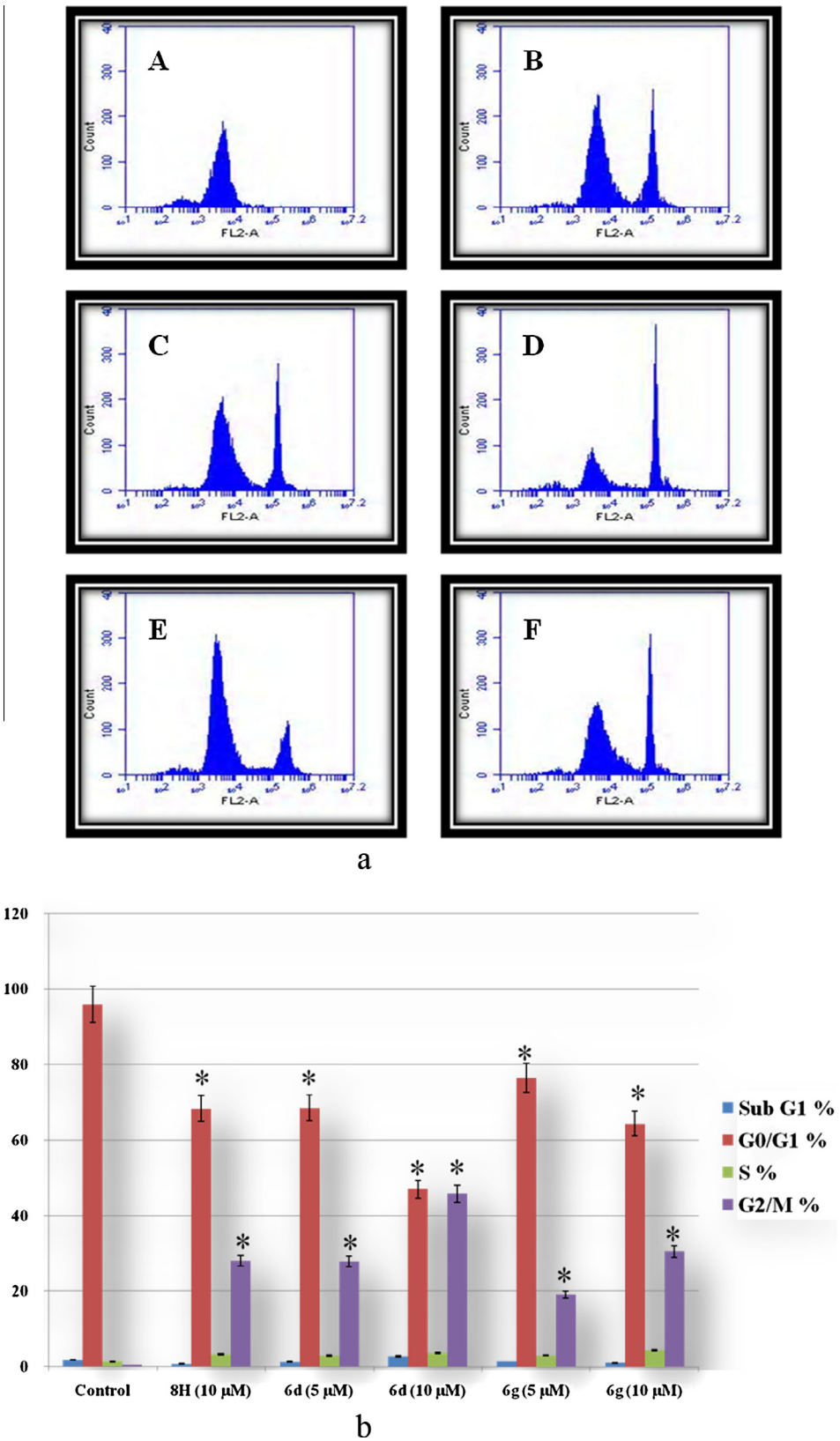


Figure 2 Flow cytometric analysis in DU-145 cells after treatment with compounds **6d** and **6g**. (**a**) A: Untreated control cells (DU-145), B: **8H** (10 μM), C: **6d** (5 μM) and D: **6d** (10 μM), E: **6g** (5 μM) and F: **6g** (10 μM); (**b**) Bar chart showing the % of cells in different phases of cell cycle after treatment with compounds **8H**, **6d** and **6g** for 48 h. Values are mean ± S.E. of three experiments. Statistical analysis was performed using GraphPad Prism software version 5.01 ($^{*}p < 0.05$ vs control).

Table 2 Distribution of DU-145 cells in various phases of cell cycle.

Compounds conc (μM)		Distribution (%)			
		Sub G1%	G0/G1%	S%	G2/M%
A: Control		1.82	95.93	1.36	0.50
B: 8H	(10 μM)	0.77	68.41	3.36	28.07
C: 6d	(5 μM)	1.25	68.62	3.00	27.85
D: 6d	(10 μM)	2.84	47.07	3.62	45.91
E: 6g	(5 μM)	1.38	76.56	3.04	19.18
F: 6g	(10 μM)	1.00	64.43	4.39	30.52

and 10 μM concentrations. After 48 h of drug treatment, cells from the supernatant and adherent monolayer cells were harvested by trypsinization and washed with PBS at 5000 rpm. Then the cells were stained with Annexin VFITC and propidium iodide using the Annexin-V-FITC apoptosis detection kit (Sigma Aldrich). Flow cytometry was performed for this study as described earlier (Browne et al., 1991).

4. Results and discussions

4.1. Chemistry

Synthetic strategies for the preparation of cinnamido-propionone conjugates are depicted in Schemes 1 and 2. Initially, substituted nitro cinnamic acids (**8a, 8b** and **14a, 14b**) were converted into their corresponding acid chlorides by reacting with oxaloyl chloride in dry CH_2Cl_2 at 0 °C for 3 h. These corresponding acid chlorides were then coupled with trimethoxy aniline in triethylamine as a base at 0 °C for 3 h to afford the corresponding nitro cinnamide derivatives (**9a, 9b** and **15a, 15b**) in excellent yields (83–89%). These nitro cinnamides are reduced by using zinc ammonium formate in methanol to obtain the amine derivatives (**10a, 10b** and **16a, 16b**). The substituted phenylpropynones **13a–e** that are required as another precursor were obtained by the reaction of aldehydes (**11a-e**) upon treatment with ethynyl magnesium bromide (0.5 M) in THF (0 °C to room temperature) for 3–4 h to produce aryl-2-propyn-1-ols (**12a-e**). Oxidation of **12a-e** with 2-iodoxybenzoic acid (IBX) in dimethyl sulfoxide (DMSO) gave the substituted phenylpropynones (**13a-e**). The synthesis of the desired cinnamido-propionone conjugates (**4a-h** and **7a-f**) was carried out by exposing alkynes to the cinnamides in ethanol for 3 h to afford them in good yields, and their structures were confirmed by ^1H and ^{13}C NMR, HRMS, and IR spectral analysis.

4.2. Biology

4.2.1. Cytotoxic activity

Preliminary screening of the synthesized conjugates (**4–6a-h** and **7a-f**) was performed to evaluate their cytotoxic potential against a panel of selected human cancer cell lines like MCF-7 (breast), A549 (lung) DU-145 (prostate) and HeLa (cervical) by using MTT assay (Vichai and Kirtikara, 2006) as shown in Table 1. Among the series, compounds **6d** and **6g** showed significant cytotoxic activity against human prostate cancer cell line (DU-145), as such this cell line was chosen for subsequent studies.

The results of this cytotoxicity data expressed as IC_{50} values in comparison with **8H** are summarized in Table 1. Interestingly, these conjugates showed considerable cytotoxic activity against most of the cell lines with micromolar range. Among the series, compounds **6d** and **6g** showed significant cytotoxic activity than **8H** against human prostate cancer cell line (DU-145), as such this cell line was chosen for subsequent studies such as inhibition of tubulin polymerization as well as apoptosis induction (see Fig. 1).

Table 3 Inhibition of tubulin polymerization (IC_{50}) of compounds **6d**, **6g** and **8H**.

Compound	IC_{50}[a] ± SD (in μM)
6d	8.98 ± 0.31
6g	9.57 ± 0.12
8H	10.11 ± 0.64
Nocodazole	2.09 ± 0.52

[a] Concentration of drug to inhibit 50% of tubulin assembly.

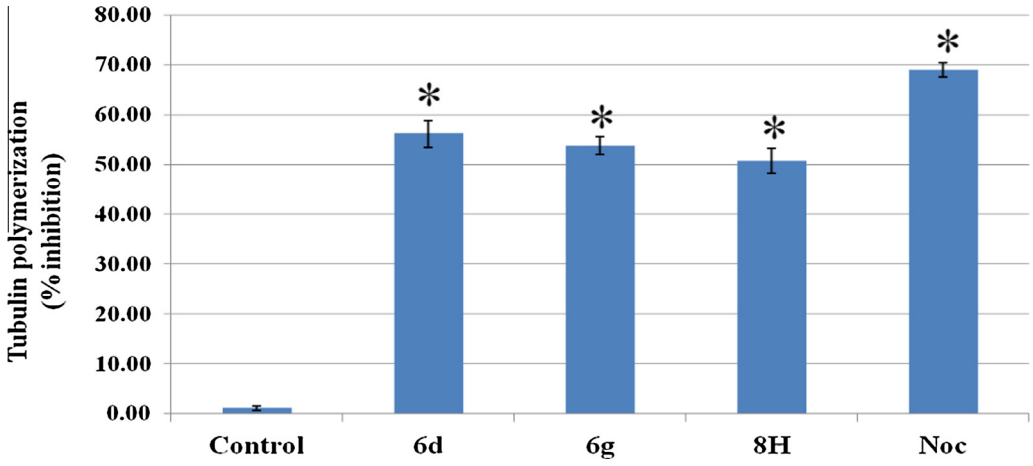
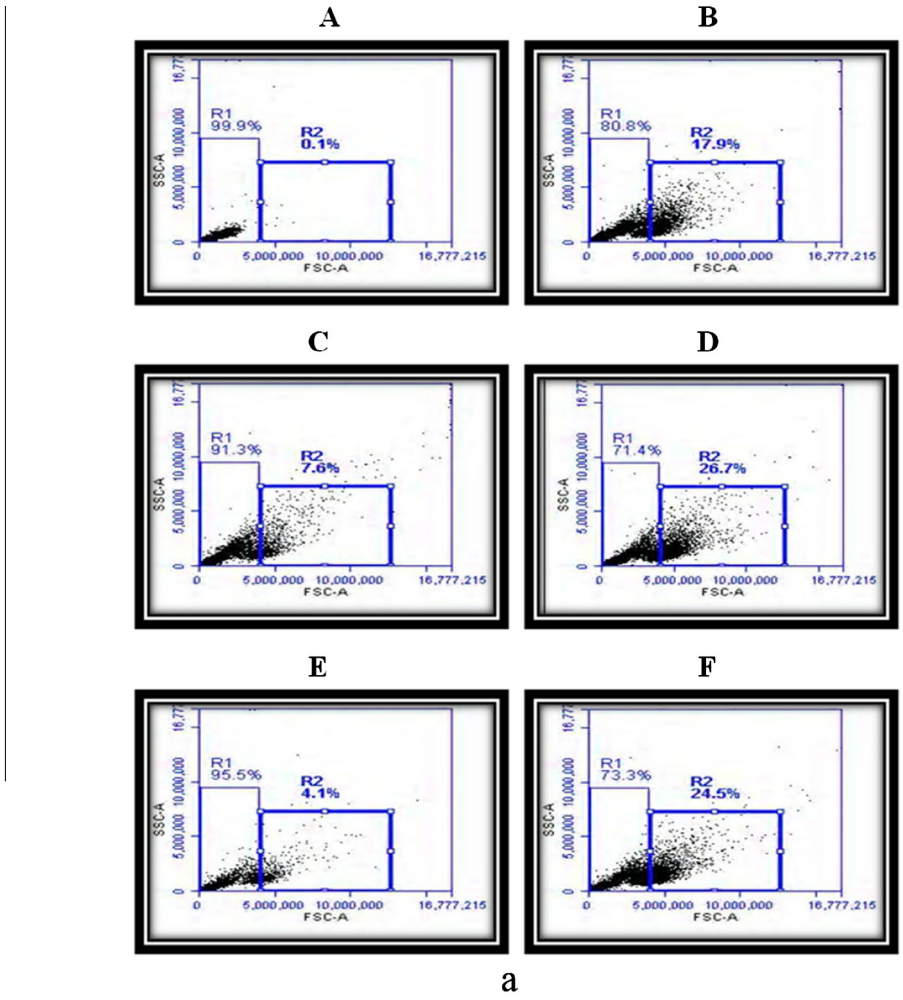


Figure 3 Effect of conjugates on tubulin polymerization: tubulin polymerization was monitored by the increase in fluorescence at 360 nm (excitation) and 420 nm (emission) for 1 h at 37 °C. Values indicated are the mean ± SD of two different experiments performed in triplicate ($^*p < 0.05$ vs control).



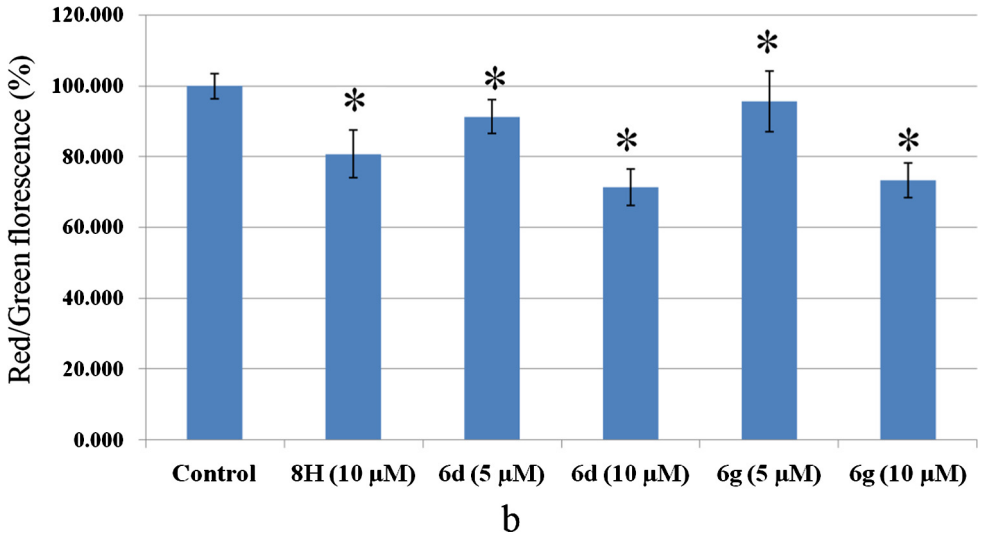


Figure 4 Compounds **6d**, **6g** and **8H** trigger mitochondrial injury. Drops in membrane potential ($\Delta\Psi m$) were assessed by JC-1 staining of DU-145 cells treated with test compound and samples were then subjected to flow cytometry analysis on a FACScan (Becton Dickinson) in the FL1, FL2 channel to detect mitochondrial potential. (**a**) A: Untreated control cells (DU-145), B: Cinnamide (10 µM), C: **6d** (5 µM) and D: **6d** (10 µM), E: **6g** (5 µM) and F: **6g** (10 µM); (**b**) Bar chart showing the ratio of red/green fluorescence. Values are mean ± S.E. of three experiments. Statistical analysis was performed using GraphPad Prism software version 5.01 ($^{*}p < 0.05$ vs control).

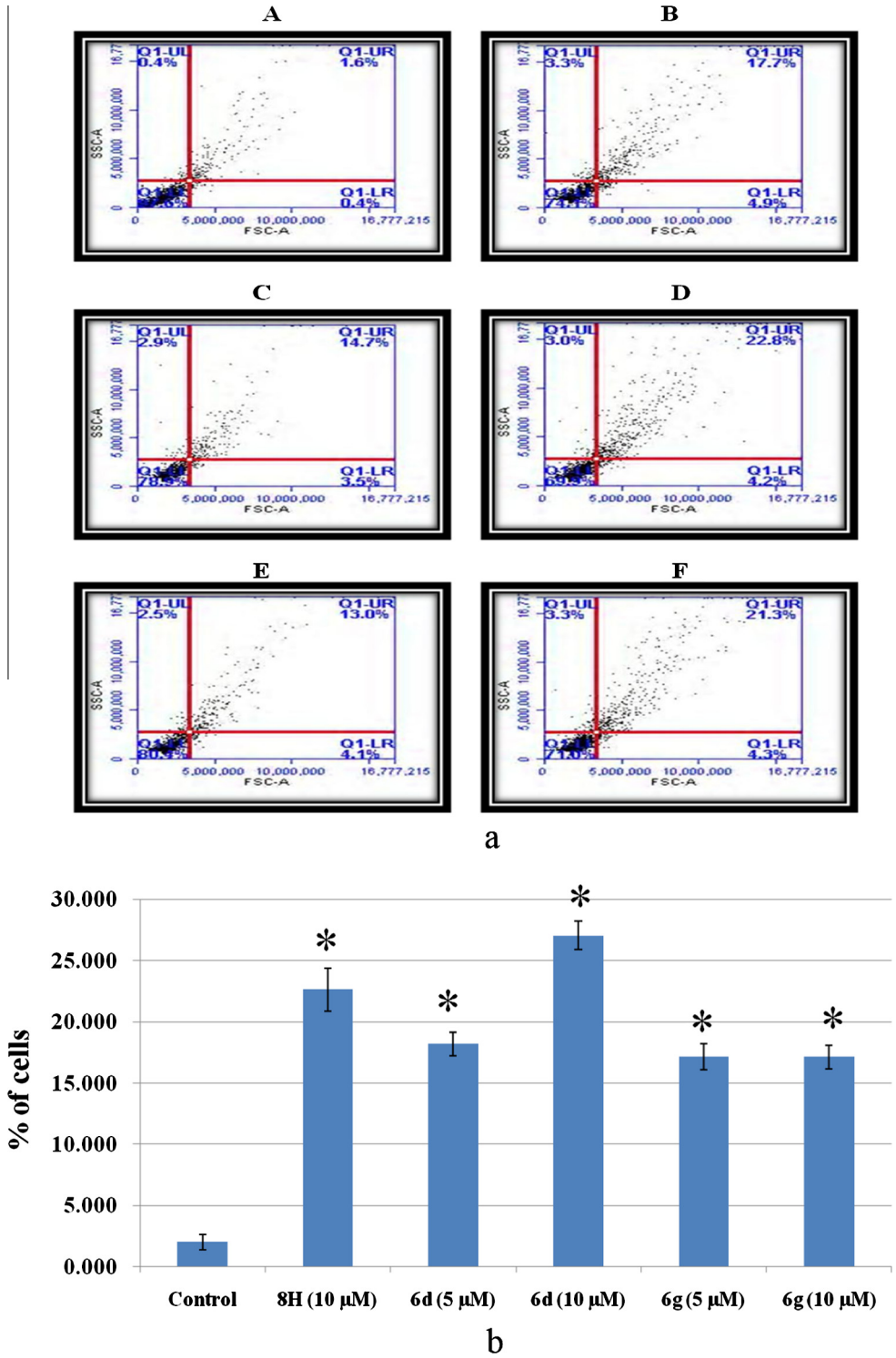


Figure 5 Annexin V-FITC staining. (**a**) A: Untreated control cells (DU-145), B: **8H** (10 µM), C: **6d** (5 µM) and D: **6d** (10 µM), E: **6g** (5 µM) and F: **6g** (10 µM); (**b**) Bar chart showing the % of apoptosis in DU-145 cells after treatment with compounds 8H, 6d and 6g for 48h. Values are mean ± S.E. of three experiments. Statistical analysis was performed using GraphPad Prism software version 5.01 ($^{*}p < 0.05$ vs control).

4.2.2. Cell cycle analysis

Many anticancer compounds exert their growth inhibitory effect either by arresting the cell cycle at a particular checkpoint of the cell cycle or by induction of apoptosis or a combined effect of both cycle block and apoptosis (Chan et al., 2010; Shen et al., 2009). The *in vitro* screening results revealed that compounds **6d** and **6g** showed significant cytotoxic activity against human prostate cancer cell line (DU-145). There-

Table 4 Distribution of apoptotic cells in Annexin-V FITC experiment.

Sample	UL%	UR%	LL%	LR%
A: Control	0.38	1.60	97.60	0.41
B: 8H(10 μM)	3.34	17.68	74.06	4.92
C: 6d (5 μM)	2.87	14.67	78.93	3.52
D: 6d (10 μM)	3.04	22.82	69.63	4.22
E: 6g (5 μM)	2.51	13.00	80.37	4.13
F: 6g (10 μM)	3.34	21.31	71.04	4.30

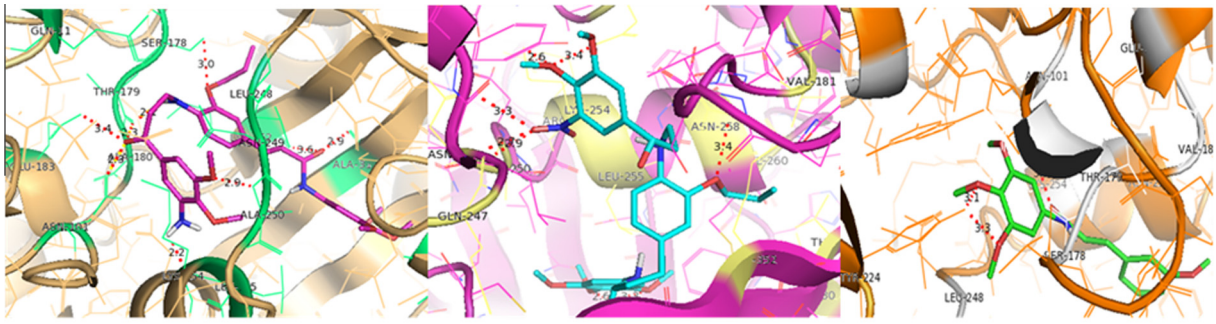


Figure 6 Superposition of binding modes of **6d** and **6g** (A and B) into colchicine binding site along with the standard **8H** (C).

fore, it was considered of interest to understand whether this inhibition of cell growth was on account of cell cycle arrest. In this study DU-145 cells were treated with these compounds at 5 and 10 μM concentrations for 48 h, and the data obtained clearly indicated that these compounds arrested the cell cycle at G2/M phase as shown in Fig. 2 and Table 2.

4.2.3. Effect on tubulin polymerization

In general G2/M cell cycle arrest is strongly associated with the inhibition of tubulin polymerization (Kanthou et al., 2004) and since conjugates **6d** and **6g** cause cell cycle arrest at G2/M phase, it was considered of interest to investigate their microtubule inhibitory function. Tubulin subunits are known to heterodimerize and self-assemble to form microtubules in a time dependent manner. The progression of tubulin polymerization was thus examined by monitoring the increase in fluorescence emission at 420 nm at 5 μM concentration (excitation wavelength is 360 nm) in 384 well plate for 1 h at 37 °C with and without the conjugates **6d, 6g 8H,** and nocodazole. These conjugates significantly inhibited tubulin polymerization by 56.13, 53.74 and 50.72% and 68.92% respectively as seen from Fig. 3. This was followed by the evaluation of IC_{50} values for these conjugates and the results are shown in Table 3. It is observed that these conjugates **6d, 6g** and **8H,** nocodazole showed tubulin-assembly inhibition with IC_{50} values of 8.98, 9.57 and 10.11, 2.09 μM respectively.

4.2.4. Measurement of mitochondrial membrane potential (ΔΨm)

The maintenance of mitochondrial membrane potential ($\Delta\Psi m$) is significant for mitochondrial integrity and bioenergetic function (Gonda et al., 2008). Mitochondrial changes, including loss of mitochondrial membrane potential ($\Delta\Psi m$), are key events that take place during drug-induced apoptosis. Mitochondrial injury by **6d**, **6g** and **8H** was evaluated by detecting drop in mitochondrial membrane potential ($\Delta\Psi m$). In this study we have investigated the involvement of mitochondria in the induction of apoptosis by these conjugates. After 48 h of drug treatment with these conjugates, it was observed that the mitochondrial membrane potential ($\Delta\Psi m$) of DU-145 cells reduced as assessed by JC-1 staining (Fig. 4).

4.2.5. Annexin V-FITC for apoptosis

The apoptotic effect of **6d** and **6g** in comparison to **8H** was further evaluated by Annexin V FITC/PI (AV/PI) dual staining assay to examine the occurrence of phosphatidylserine externalization and also to understand whether it is due to physiological apoptosis or nonspecific necrosis (Zhu et al., 2010). In this study DU-145 cells were treated with these conjugates for 48 h at 5 and 10 μM concentrations to examine the apoptotic effect. It was observed that these conjugates showed significant apoptosis against DU-145 cells as shown in Fig. 5. Results indicated that conjugates **6d** and **6g** showed 18.19 and 17.13% of apoptosis at 5 μM concentration, whereas they exhibited 27.04 and 25.61% of apoptosis at 10 μM concentration respectively for 48 h, whereas **8H** showed 22.6% apoptosis at 10 μM concentration when compared to untreated control cells as shown in Table 4.

4.2.6. Molecular modelling studies: (Cormier et al., 2008)

We know that cinnamides are often key pharmacophores prevalent in many anticancer leads which act through inhibiting tubulin polymerization. Moreover, this has been experimentally proven which is discussed in the previous sections. Therefore, molecular docking studies were performed to get an insight into binding modes of the promising conjugates **6d** and **6g** with the tubulin. The coordinates of the protein structure cocrystallized with colchicine (PDB ID: 3E22) (Cormier

et al., 2008) were obtained from Protein Data Bank. Docking studies were performed using AutoDock 4.2 (Morris et al., 2009) and the visualization was done using Pymol, v. 0.99 (Delano, 2002). Docking pose shown in Fig. 6 indicates that the trimethoxy phenyl ring of the cinnamide moiety in both **6d** and **6g** was buried in the hydrophobic region of the colchicine binding site in the β chain, similar to that of colchicine. Both the compounds were found to interact extensively with the neighbouring amino acid residues such as Val 238, Cys 241, Leu 248, Asn 249, Ala 250, Leu 255, Val 318, Ileu 378 and Ala 354. Interestingly, **6d** and **6g** displayed several hydrogen bonding interactions with the amino acid residues in proximity that include Thr 179, Asn 101, Lys 254, Ala 317 and Tyr 202, Ala 250, Lys 254, Asn 249 respectively. It is important to note that these amino acid residues form hydrogen bond with all the major functionalities and rings contained in **6d** and **6g**. Besides this both the compounds established a series of secondary interactions such as van der Waal and polar interactions with some other amino acid residues. On the other hand, the standard **8H** was found to establish only two hydrogen bonds with Tyr 224 in addition to some hydrophobic and polar interactions with the surrounding amino acid residues. The different binding modes of **6d** and **6g** than **8H** may be due to the longer keto-enamine substitution on the cinnamide pharmacophore. Therefore, this study infers that compounds **6d** and **6g** bind to the colchicine binding pocket of tubulin better than the standard which is in correlation with the cytotoxicity data represented in Table 1.

5. Conclusion

In the present study, we have synthesized cinnamido-propionone conjugates and evaluated them for their cytotoxic potential. Among them, conjugates **6d** and **6g** showed significant cytotoxic activity against human prostate cancer cell line, (DU-145). The flow cytometric analysis revealed that these conjugates cause cell cycle arrest at G2/M phase. Furthermore, they effectively inhibited microtubule assembly. Moreover, the triggering of the apoptotic cell death after mitotic arrest was investigated by mitochondrial membrane potential and Annexin V FITC assays suggested that these conjugates induced apoptosis. The molecular modelling study carried out on the colchicine binding site of tubulin demonstrated that these molecules are involved in a series of interactions with the protein thereby binding well with the tubulin. Therefore, the work reported herein could be considered of significant importance to provide valuable insights in the development of newer leads for the treatment of cancer.

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