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# Synthesis and biological evaluation of some novel triazole hybrids of curcumin mimics and their selective anticancer activity against breast and prostate cancer cell lines[#]

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## Abstract:

The anti-cancer property of curcumin, an active component of turmeric, is limited due to its poor solubility, stability and bioavailability. To enhance its efficacy, we designed a novel series of twenty-four monocarbonyl curcumin analogue–1,2,3-triazole conjugates and evaluated their anti-cancer activity towards endocrine related cancers. The new compounds (**17-40**) were synthesized through CuAAC click reaction and SAR analysis carried out. Out of these all, compound **17** showed most significant anti-cancer activity against prostate cancer cells with $IC_{50}$ values of 8.8μM and 9.5μM in PC-3 and DU-145 cells respectively. Another compound **26** showed significant anti-cancer activity against breast cancer cells with $IC_{50}$ of 6 μM, 10 μM and 6.4 μM in MCF-7**,** MDA-MB-231 and 4T1 cells, respectively while maintaining low toxicity towards non-cancer originated cell line, HEK-293. Compound **17** and **26** arrested cell cycle and induced mitochondria-mediated apoptosis in cancer cells. Further, both of these compounds significantly down-regulated cell proliferation marker (PCNA), inhibited activation of cell survival protein (Akt phosphorylation), upregulated pro-apoptotic protein (Bax) and down-regulated anti-apoptotic protein (Bcl-2) in their respective cell lines. In addition, *in vitro* stability, solubility and plasma binding studies of the compound **17** and **26** showed them to be metabolically stable. Thus, this study identified two new curcumin monocarbonyl-1,2,3-triazole conjugate compounds with more potent activity than curcumin against breast and prostate cancers.

***Keywords:*** Curcumin mimics, 1,2,3-Triazole, Endocrine cancers, AKT-pathway, Hybridization.

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Cancer is a group of heterogeneous disease, commonly characterized by uncontrolled cell division that can further progress towards mortality through cancer cells acquiring ability to invade other vital organs. After cardiovascular disease, cancer is the most life threatening disease that causes one out of eight deaths worldwide. An estimated 13.2 million cancer related deaths and ~21.4 million new cancer cases are expected by 2030 [1]. National Institutes of Health (NIH) has projected medical expenditures to reach $158 billion in the year 2020 with total cost estimates of $895 billion worldwide. The highest medical costs associated with the two endocrine related cancers–breast cancer ($16.5 billion) in women and prostate cancer ($12 billion) in men [2]. Among women, breast cancer is the leading cause of cancer deaths worldwide, with nearly 1.67 million new cases diagnosed accounting more than 25% of all the cancers in 2012. On the other hand, prostate cancer is the second leading cause of cancer deaths in men, contributing to ~15% of the total number of new cases diagnosed in 2012 [3]. In cancer related deaths breast cancer and prostate cancer accounted for 6.4% and 3.7% respectively in 2012 [4]. The incidence of these two cancers has significantly increased in developed countries like United States and is constantly rising in developing countries [5]. However, significant progress has been made in diagnosis and therapeutic management of cancers in the last several years due to increased knowledge and innovations. Nevertheless, all currently available chemotherapeutic agents suffer from undesirable off-target effects and frequently develop resistance. The majority of successful anti-cancer drugs have been discovered from natural resources and development of natural product derived semi-synthetic anti-cancer agents is a promising area for any cancer drug discovery programs [6]. Curcumin, (Figure 1) an active component of *Cucuma longa* (turmeric), has not only been shown to prevent carcinogenesis, but also known to have direct therapeutic benefits in various cancers [7]. Turmeric is a culinary spice and medicine regularly consumed in Eastern Asian countries, and thought to contribute to the massive difference in the rates of prostate cancer incidences between Eastern (less than 10 per 100,000 in Asia) and Western (120 per 100,000 in Northern America) countries [8]. The anti-cancer effect of curcumin is due its moderate effect on multiple growth factor pathways in the

cell, and its multiple weak effects result into a remarkable aggregate effect, which is valuable in complex diseases like cancer [9]. However, low bioavailability and rapid plasma clearance limits clinical application of curcumin. The metabolic instability of curcumin is found to be due to the β–diketone moiety. This was successfully circumvented by replacing the β–diketone moiety with single carbonyl group to synthesize monocarbonyl curcumin analogue with improved efficacy and stability [10-13]. The anti-cancer activity of monocarbonyl curcumin analogue against endocrine related breast cancer [14] and prostate cancer is due to inhibition of AKT, HER2/neu, and signal transducer and activator of transcription 3 (STAT3) signaling pathways [15].

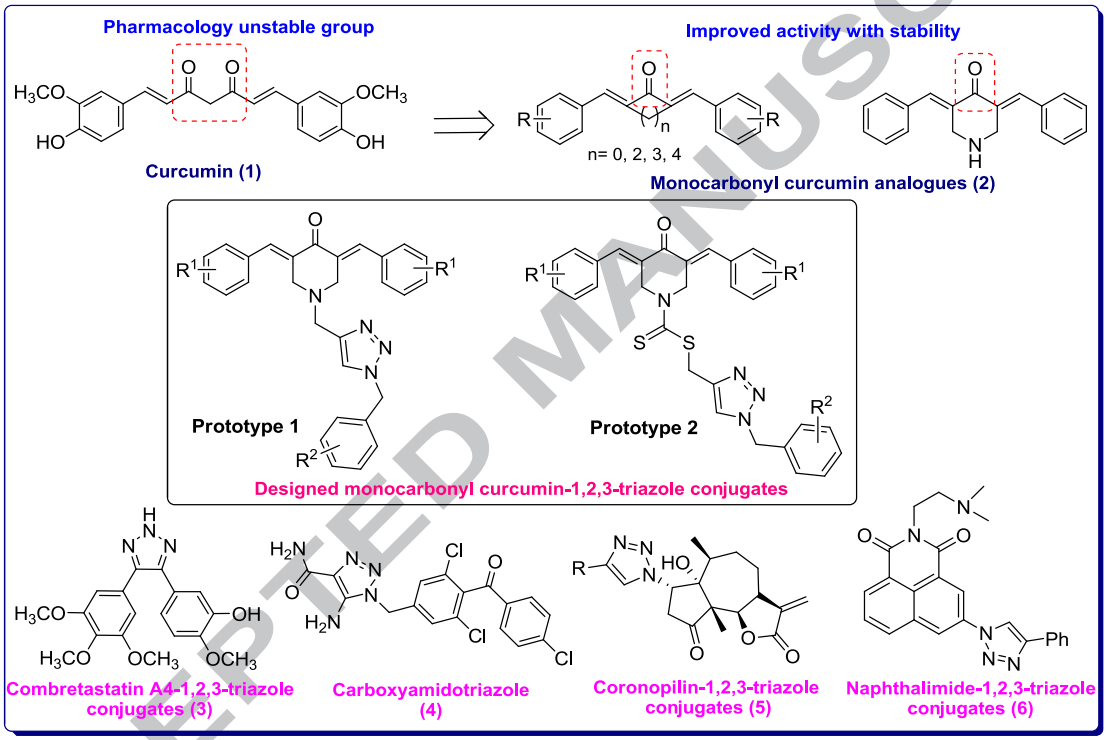


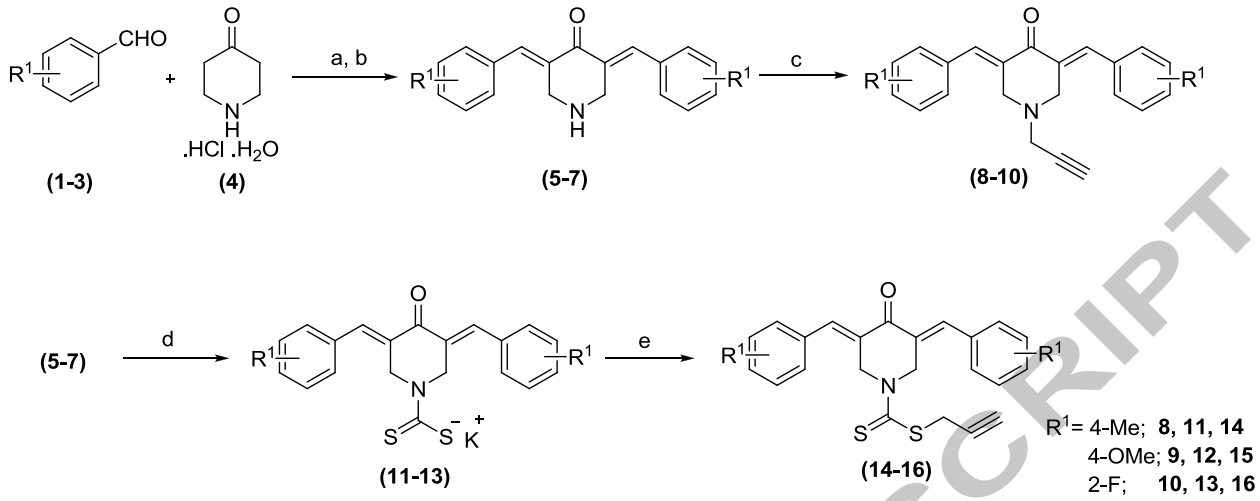

**Figure 1:** Structures of Curcumin (**1**), monocarbonyl curcumin analogues (**2**), 1,2,3-triazole containing anti cancer molecules (**3-6**) and envisaged monocarbonyl curcumin analogue–1,2,3-triazole conjugates (prototype 1 and 2).

Molecular hybridization is a common strategy used for developing new chemotherapeutic agents uniting two or more active pharmacophores into a conjugated single molecule and thereby creating distinct novel chemical entities [16-22]. These novel conjugates may modulate multiple cellular pathways simultaneously through synergistic action with minimum redundant side effects compared to single-target drugs [23]. 1,2,3–triazoles conjugation increases metabolic stability, water solubility and infuse capability to readily interact with biological targets through

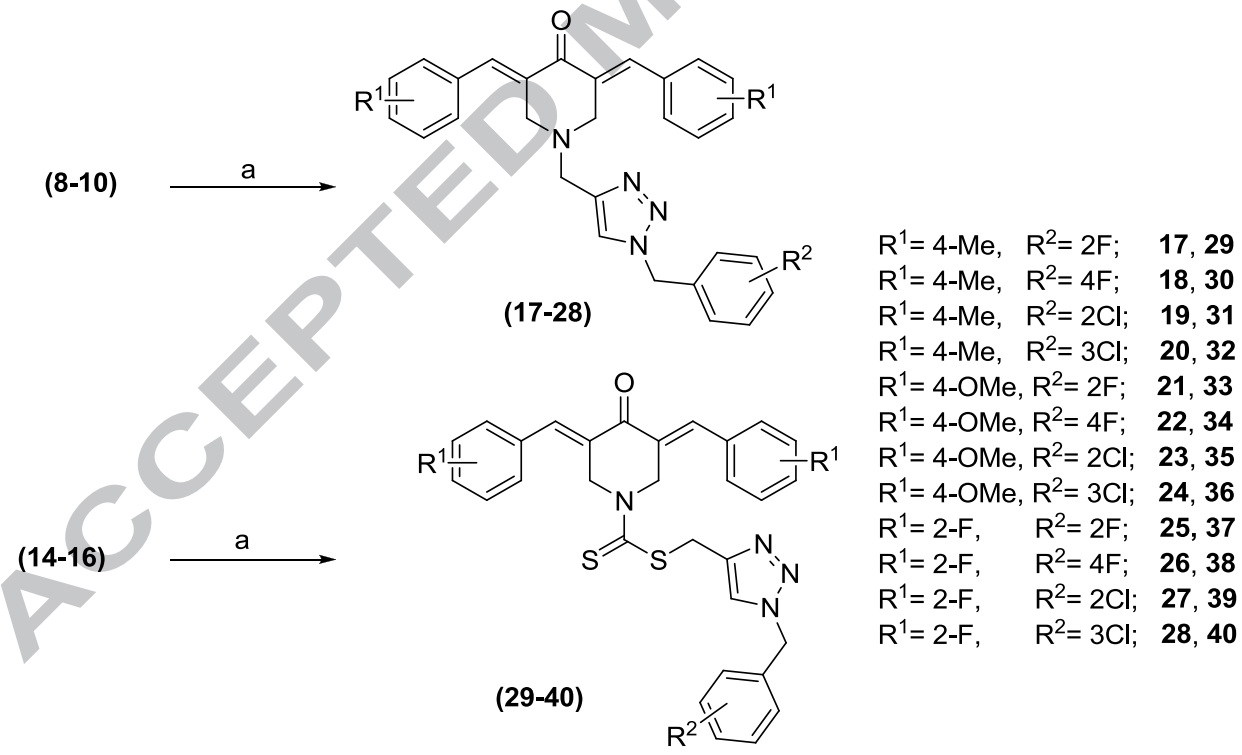
hydrogen bonding and dipole interactions [24]. Their synthetic accessibility through click chemistry and widely reported pharmacological effects [25-28] makes it appealing to anti-cancer drug design. In fact, 1,2,3–triazoles conjugates have already been reported to exhibit potent anti-cancer activity [29-33] and carboxyamidotriazole [34], a 1,2,3-triazole bearing anti-cancer drug, is now available in the market (Fig. 1). On the other hand, dithiocarbamates are reported as effective anti-cancer agents [35-37].

As part of our ongoing interest in developing novel hybrid therapeutic agents against endocrine related cancers [38] and generating better synthetic analog of curcumin, we designed and synthesized a novel series of hybrid scaffolds by combining monocarbonyl curcumin analogue with 1,2,3-triazoles (fig. 1, prototype1). In prototype 2, the dithiocarbamate moiety was incorporated to achieve better biological activity. Compounds were synthesized and biologically evaluated against breast cancer and prostate cancer cell lines using MTT assay along with curcumin as positive control. This letter reports the effect of these new analogs on cancer cell viability, cell cycle and apoptosis along with their SAR.

*Synthesis:* The general procedure for the synthesis of the intermediates (3*E*,5*E*)–3,5–bis(substitutedbenzylidene)–1–(prop–2–ynyl)piperidin–4–one (**8–10**) and (3*E*,5*E*)–prop–2–ynyl 3,5–bis(substitutedbenzylidene)–4–oxopiperidine–1–carbodithioate (**14–16**) have been illustrated in Scheme 1. (3*E*,5*E*)–3,5–bis(substitutedbenzylidene)piperidin–4–one (**5-7**) were synthesized by reacting substituted benzaldehydes (**1-3**) with 4–piperidinone hydrochloride hydrate (**4**) in presence of small amount of conc.HCl in glacial acetic acid at room temperature followed by basifying with potassium carbonate in acetone:water (5:1) in good yields [39]. Furthermore, (3*E*,5*E*)–3,5–bis(substitutedbenzylidene)–1–(prop–2–ynyl)piperidin–4–one (**8–10**) were obtained by treating **5–7** with propargyl bromide in acetone at room temperature. The synthesis of hitherto unknown dithiocarbamate potassium salts (**11–13)** were obtained by reacting **5–7** with carbon disulfide, aq. KOH in DCM at 0 $^{o}$C. **11–13** further gave rise intermediates (3*E*,5*E*)–prop–2–ynyl 3,5–bis(substitutedbenzylidene)–4–oxopiperidine–1–carbodithioate (**14–16)** by reacting with propargyl bromide in water:acetone (5:1) at 10–15 $^{o}$C.




**Scheme 1:** *Reagents and conditions*: (a) glacial acetic acid, Conc. HCl, rt, 48 h; (b) $K_2CO_3$, acetone:water (5:1), 20 $^oC$, 2 h; (c) propargyl bromide (1 eq.), acetone, $K_2CO_3$, rt, 5–6 h; (d) carbon disulfide (1.3 eq.), aq. KOH, DCM, 0 $^oC$, 2 h; (e) propargyl bromide (1 eq.), water:acetone (5:1), 10–15 $^oC$, 1 h.




**Scheme 2:** *Reagents and conditions:* (a) $CuSO_4.5H_2O$ (0.05 eq.), sodium ascorbate (0.1 eq.), THF:water (1:1), rt, 1–1.5 h.

The final compounds, (3*E*,5*E*)–1–((1–(substitutedbenzyl)–1*H*–1,2,3–triazol–4–yl)methyl)–3,5–bis(substitutedbenzylidene) piperidin–4–ones (prototype 1; **17–28**) and (3*E*,5*E*)–(1–(substitutedbenzyl)–1*H*–1,2,3–triazol–4–yl)methyl 3,5–bis(substitutedbenzylidene)–4–oxopiperidine–1–carbodithioates (prototype 2; **29–40**) were obtained by reacting a mixture of **8–10** or **14–16** (scheme 1) with substituted benzyl azides in presence of catalytic amount of $CuSO_4.5H_2O$ and sodium ascorbate in a mole ratio of 2:2:0.2:0.1 in 1:1 volumes of $THF/H_2O$ for 1–1.5 h in good yields.

*Biological Evaluation*: The anti-cancer activity of the two prototypes synthesized (**17–40**) were evaluated against human breast cancer cell lines (MCF–7 and MDA–MB–231), mouse mammary tumor cell line (4T1), human prostate cancer cell lines (PC–3 and DU–145) and non–cancer originated human embryonic kidney cell line (HEK–293) using MTT assay. Cells were treated with compounds for 24 h and curcumin was used as a standard drug. Interestingly as illustrated in table 1, all the compounds except **34**, **35** and **40** induced loss of cell viability of breast cancer cells. Out of the twenty four compounds evaluated, sixteen compounds (**17-21, 24-28, 30, 33, 36-39**) exhibited anti-cancer activity with $IC_{50}$ 6.00 µM–100 µM against MCF–7 cells. Of these fourteen compounds (**17-20, 25-28, 30, 33** and **36-39**) demonstrated 1 to 13.8 fold higher activity than standard curcumin ($IC_{50}$ 83.1 µM) at $IC_{50}$ values ranges from 6 µM–81.6 µM. Whereas against MDA–MB–231 cells, nineteen compounds (**17-22, 24-32, 36-39**) were active ($IC_{50}$ 9.00µM–100.7µM). Among these fourteen compounds **17-22, 25-28, 30** and **36-38** showed up to 8.3 fold higher activity than curcumin ($IC_{50}$ 75.3 µM). In case of 4T1 mouse mammary tumor cells, all the test compounds except **34, 35** and **40** were active at $IC_{50}$ 5.3µM–69.7µM of which sixteen compounds (**17, 19-23, 25-31** and **37-39**) were more active (up to 9.3 fold) than standard curcumin. Compound **26** showed most significant activity against all the breast cancer cell lines. The activity data against prostate cancer cell line PC-3 revealed that seventeen compounds (**17-28, 30, 33-35, 39** and **40**) were active with $IC_{50}$ 7.1–80.0 µM while against DU–145 cells eighteen compounds (**17-30, 33, 34, 38** and **39**) were active at $IC_{50}$ values 4.3–74.6 µM. Ten compounds **17, 19, 20, 23-28** and **33** against PC–3 cells and another ten compounds (**17, 19-21, 23-28, 30**) against DU-145 cells showed up to 5.7 and 9.5 fold better activity than curcumin (PC–3, 39.0 µM and DU-145, 41 µM) respectively. On the other hand, twenty compounds (**17–30, 33–35, 38** and **40**) exhibited cell growth inhibitory activity against prostate cancer cells either

PC-3 or DU–145 while fifteen (**17**–**28**, **30, 33** and **34**) of these were active against both the cell lines.

**Table 1.** *In vitro* anti-cancer activity ($IC_{50}$)[a] of all the synthesized compounds (**17-40**) in µM.

| Comp. | $R^1$ | $R^2$ | Breast cancer cell lines | | | Prostate cancer cell lines | | Normal cell line |
|---|---|---|---|---|---|---|---|---|
| | | | MCF-7 | MDA-MB-231 | 4T1 | PC-3 | DU-145 | HEK |
| **17** | 4-$CH_3$ | 2-F | 28.7 ± 1.6 | 10 ± 1.1 | 18.0 ± 0.9 | 8.8 ± 0.2 | 9.5 ± 0.2 | >100 |
| **18** | 4-$CH_3$ | 4-F | 29 ± 1.8 | 26.8 ± 1.1 | 57.2 ± 1.8 | 65.6 ± 2.1 | 49.7 ± 1.3 | ND |
| **19** | 4-$CH_3$ | 2-Cl | 66.7 ± 2.0 | 29 ± 0.6 | 23.2 ± 1.7 | 8.9 ± 0.5 | 10.5 ± 0.1 | 91.5 ± 0.4 |
| **20** | 4-$CH_3$ | 3-Cl | 10 ± 1.1 | 9 ± 0.5 | 29.3 ± 1.1 | 29.3 ± 1.8 | 33.1 ± 1.2 | 73.5 ± 1.5 |
| **21** | 4-$OCH_3$ | 2-F | ≥100 | 67.2 ± 0.5 | 39.4 ± 1.6 | 39.9 ± 1.5 | 32.6 ± 0.9 | >100 |
| **22** | 4-$OCH_3$ | 4-F | >100 | 70.7 ± 0.6 | 49.3 ± 1.5 | 67.5 ± 2.9 | 59.4 ± 1.2 | ND |
| **23** | 4-$OCH_3$ | 2-Cl | >100 | >100 | 11.4 ± 0.9 | 36.3 ± 0.9 | 17.8 ± 1.7 | ND |
| **24** | 4-$OCH_3$ | 3-Cl | 99 ± 1.7 | 98 ± 0.9 | 56.6 ± 1.9 | 32.5 ± 1.1 | 15.4 ± 0.7 | >100 |
| **25** | 2-F | 2-F | 6 ± 1.2 | 16 ± 1.3 | 6.5 ± 1.2 | 19.7 ± 1.0 | 10.6 ± 0.2 | 41.2 ± 1.3 |
| **26** | 2-F | 4-F | 6.0 ± 1.0 | 10.0 ± 1.6 | 6.4 ± 1.9 | 19.4 ± 0.5 | 8.7 ± 0.3 | 46.0 ± 0.7 |
| **27** | 2-F | 2-Cl | 10 ± 1.6 | 16.5 ± 0.4 | 5.3 ± 0.9 | 7.1 ± 0.3 | 7.5 ± 0.6 | 33.2 ± 2.0 |
| **28** | 2-F | 3-Cl | 12 ± 1.0 | 12 ± 0.3 | 6.1 ± 0.5 | 6.8 ± 0.3 | 4.3 ± 0.6 | 33.1 ± 1.0 |
| **29** | 4-$CH_3$ | 2-F | >100 | 91.5 ± 1.3 | 28.4 ± 1.6 | >80 | 80 ± 2.2 | ND |
| **30** | 4-$CH_3$ | 4-F | 81.6 ± 1.3 | 45.6 ± 0.9 | 29.3 ± 1.1 | 45.5 ± 4.7 | 38.8 ± 4.9 | ND |
| **31** | 4-$CH_3$ | 2-Cl | >100 | 93.2 ± 1.7 | 49.3 ± 1.5 | >80 | >80 | ND |
| **32** | 4-$CH_3$ | 3-Cl | >100 | 83 ± 1.9 | 53.4 ± 1.3 | >80 | >80 | ND |
| 33 | 4-$OCH_3$ | 2-F | 79.4 ± 1.3 | >100 | 49.9 ± 1.2 | 31.5 ± 1.2 | 54.5 ± 1.8 | ND |
| **34** | 4-$OCH_3$ | 4-F | >100 | >100 | >100 | 45.6 ± 0.8 | 74.6 ± 1.9 | ND |
| **35** | 4-$OCH_3$ | 2-Cl | >100 | >100 | >100 | 60.1 ± 1.4 | >80 | ND |
| **36** | 4-$OCH_3$ | 3-Cl | 32 ± 1.1 | 32 ± 0.5 | 69.7 ± 2.0 | >80 | >80 | >100 |
| **37** | 2-F | 2-F | 51.6 ± 1.6 | 57.6 ± 1.5 | 30.7 ± 1.2 | >80 | >80 | ND |
| **38** | 2-F | 4-F | 56.9 ± 1.3 | 58.8 ± 1.1 | 9.9 ± 1.5 | >80 | 35.7 ± 3.5 | 51.3 ± 1.3 |
| **39** | 2-F | 2-Cl | 78.7 ± 2.1 | 100 ± 2.0 | 43.6 ± 0.8 | >80 | 46.3 ± 1.0 | ND |
| **40** | 2-F | 3-Cl | >100 | >100 | >100 | 80 ± 1.1 | >80 | ND |
| **Standards** | **Curcumin** | | 83.1 ± 4.4 | 75.3 ± 2.8 | 49.4 ± 1.4 | 39 ± 3.2 | 41 ± 2.9 | 94.1 ± 1.4 |
| | **Doxorubicin** | | 0.13±0.22 | 1.6±0.23 | 0.99±0.13 | 1.4±0.34 | 1.6±0.21 | 7.2±1.12 |
| | **5 Fluorouracil** | | 15±.23 | 19±2.51 | 23±3.52 | 13.3±1.54 | 24.2±2.67 | 40±4.51 |
| | **Nocodazole** | | 0.42±0.32 | 1.1±1.2 | 1.3±0.23 | 2.5±1.11 | 2.2±0.54 | 5.1±1.15 |

[a]Data are expressed in terms of Mean ± SE of three independent experiments; ND- Not tested

Out of all the compounds evaluated compound **26** showed most significant anti-cancer activity against all the breast cancer cell lines. Similarly, compound **17** turned out to be the most active against all prostate cancer cell lines (Table 1). Interestingly, all the compounds did not induced significant cytotoxicity against non-cancer originated cell lines (HEK-293) suggesting specificity of the compounds towards cancer cells.

*Structure–activity relationship (SAR)*: Against MCF-7 cells the 2-fluoro substitution (**25-28**) at $R^1$ position of prototype 1 was more desirable and the order of preference was 4-methyl (**17-20**) followed by 4-methoxy (**21-24**) groups irrespective of the $R^2$ substitution. When $R^1$ is 2-fluoro (**25-28**) then a fluoro substituent (**25**, **26**) at $R^2$ position was found to be more favorable than chloro group (**27**, **28**). Against PC-3 cells the preference of substituent at $R^1$ was again 2-fluoro (**25-28**) > 4-methyl (**17-20**) > 4-methoxy (**21-24**) groups. While at $R^2$ position chloro substituent (**19**, **20**; **23**, **24**; **27**, **28**) was more desirable over fluoro substituent (**17**, **18**; **21**, **22**; **25**, **26**). 2-choloro substitution was better with 4-methyl at $R^1$ (**19**) while 3-chloro was better over 2-chloro substituent with 4-methoxy or 2-fluoro at $R^1$ (**24**, **28**). In the prototype 2 which contained an additional dithiocarbamate group between Curcumin and 1,2,3-triazole scaffolds the order of activity with respect to substituent at $R^1$ was 2-fluoro > 4-methoxy > 4-methyl groups. At $R^2$ position fluoro substitution seemed to be more favorable over chloro substitution as out of twelve compounds (**29-40**), four compounds (**30, 33, 37, 38**) with fluoro substituent were active while with chloro substitution (**36, 39**) only two were found to be active. Whereas against PC-3 cell line, at $R^1$ position 4-methoxy substituent (**33-36**) was more desirable over 4-methyl (**29-32**) and 2-fluoro groups (**37-40**). A fluoro substituent at $R^2$ position (**33**, **34**) gave much better activity with 4-methoxy at $R^1$ position. The SAR study revealed that both the ring substitutions $R^1$ (phenyl ring in monocarbonyl curcumin analogue scaffold) and $R^2$ (1,2,3-triazole scaffold) were detrimental for activity. The SAR study (table 1) illustrated that monocarbonyl curcumin analogue-1,2,3-triazole conjugate (prototype 1; **17-28**) was more desirable scaffold as compared to dithiocarbamate incorporated monocarbonyl curcumin analogue-1,2,3-triazole conjugates (prototype 2; **29-40**). This biological activity of two prototypes further supported the importance of the active scaffold in this study. It is interesting to observe that twelve compounds (**17**-**21**, **24**-**28**, **30** and **33**) were active against both PC-3 and MCF-7 cancer cells.

Out of all the compounds evaluated, compounds **17** and **26** were selected as lead molecules for further investigation due to their potent activity against prostate cancer and breast cancer cells respectively and better cytotoxicity profile towards non-malignant cell line (HEK-293). In order to investigate the effect of compounds **17** and **26** on clonogenic potential on PC-3 and MCF-7 cells respectively, cells were treated for 24 h and colony formation was assessed. Tumor is composed of heterogeneous cell types and some subpopulation may contain long term clonal renewal capacity which is often considered as a source of tumor refractoriness towards

chemotherapy. Clonogenic potential is described as the cell renewal capacity or tumorigenic potential of cancer cells after chemotherapy or radiation therapy is withdrawn. Compounds **26** efficiently inhibited MCF-7 colony formation and no colonies were seen in MCF-7 cells till 7$^{th}$ day of evaluation. Compound **17** significantly and dose-dependently retarded colony formations in PC-3 cells though complete inhibition was not observed (Fig. 2).

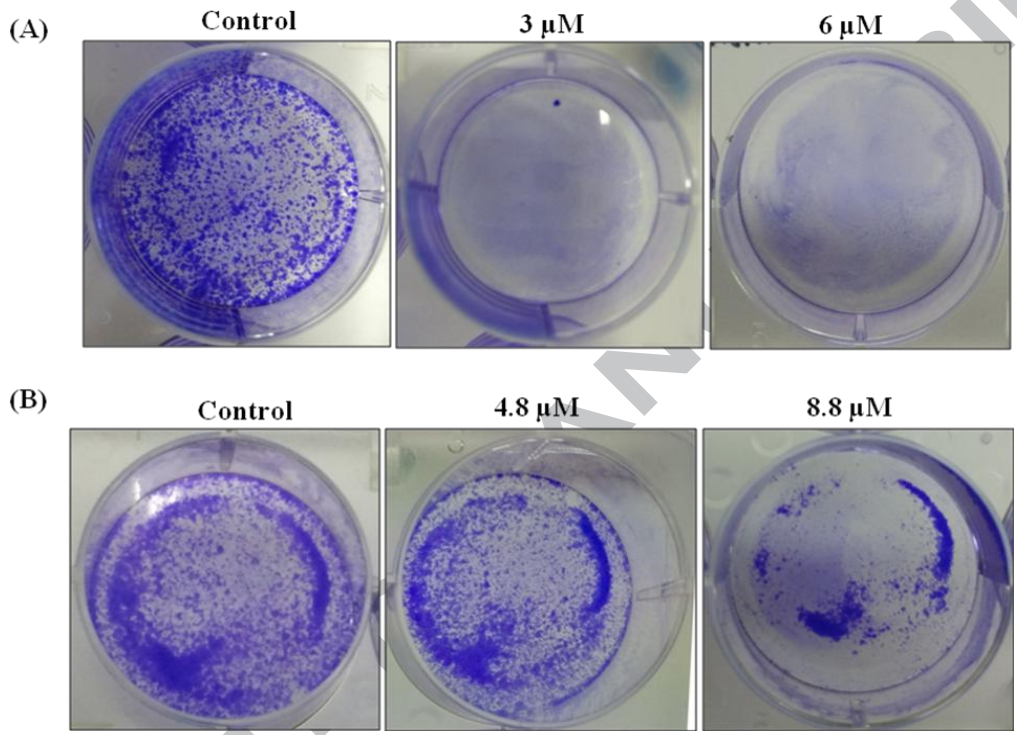



**Figure 2:** ***Effect of compounds on colony formation*** (A) MCF-7 cells were treated with different concentrations of compounds **26** for 24 h (B) PC-3 cells were treated with different concentrations of compounds **17** for 24 h. At the end of treatment, medium were replaced with complete medium and cells allowed to grow for 7 days. Colony formation was checked by staining with 0.1% crystal violet.

Further the effect of compounds **17** and **26** on cell cycle progression was assessed. Dysregulation of the cell cycle check points and over expression of proteins associated with cell cycle regulation are the hallmark of malignancy. Therefore, compounds that are able to arrest cell cycle progression have beneficial effect in cancer treatment [40]. To check the effect of most active compounds **17** and **26** on cell cycle phase distribution following treatment of 24 h, distribution of cell cycle phases was evaluated using PI staining method. Compound **26** induced significant decrease in G0/G1 population and subsequent increase in S phase dose dependently in

MCF-7 cells (Fig. 3A). Whereas, compound **17** increased the cell population at G2/M of cell cycle in PC-3 cells (Fig. 3B).

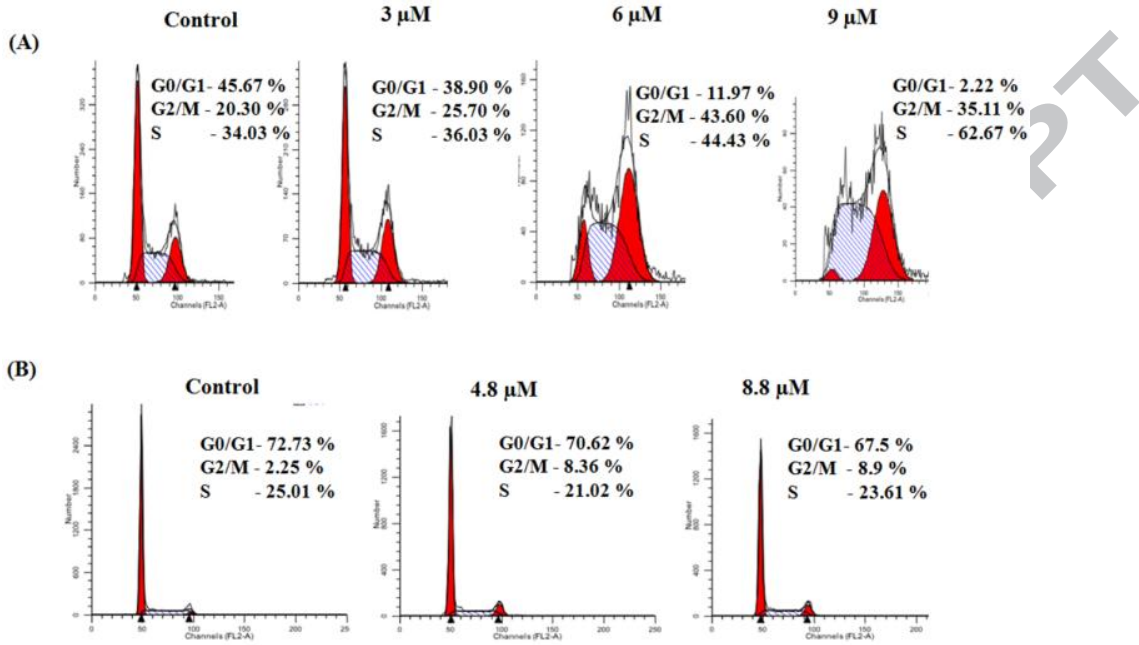



**Figure 3:** ***Effect of compound 26 and 17 on cell cycle progression in MCF-7 and PC-3 cells respectively.*** (A) MCF-7 cells were treated with compound **26** (B) PC-3 cells were treated with compound **17** for 24 h, cells were harvested, stained with PI and acquired by flow-cytometer.

In addition, phase contrast microscopy was used to evaluate the effect of compounds **26** and **17** on morphological changes on MCF-7 and PC-3 cells respectively. Results showed that both the compounds caused significant decrease in numbers as well as alteration of cellular morphology in MCF-7 as well as in PC-3 cells (Fig. 4) suggestive of cytotoxicity against cancer cells. The intrinsic morphology of cells was altered and eventually detached from surface upon exposure at higher concentrations.

Apoptosis maintain natural balance between healthy cell survival and death of excess, damaged or abnormal cells [38]. However, the balance often inclines towards abnormal cell survival in cancer due to inhibition or evasion of apoptosis. Therefore, in order to evaluate induction of apoptosis following treatment with compound **17** and **26**, AnnexinV-FITC/PI dual staining assay was carried out [44]. MCF-7 cells were treated with various concentrations of compound **26** (3µM, 6µM and 9µM) and PC-3 cells with compound **17** (4.8 µM and 8.8 µM) for 24h. There was a dose dependent increase of early apoptosis ($AnnexinV^{+}$/ $PI^{-}$) and subsequent decrease of

live cells (AnnexinV$^-$/ PI$^-$) following compound treatment in MCF-7 as well as in PC-3 cells (Fig. 5). In contrast, no significant change in necrotic population (AnnexinV$^-$/ PI$^+$) was seen with both compounds (Fig. 5). These data clearly indicate that compound **26** and **17** induced apoptosis mediated cell death in MCF-7 and PC-3 cells respectively.

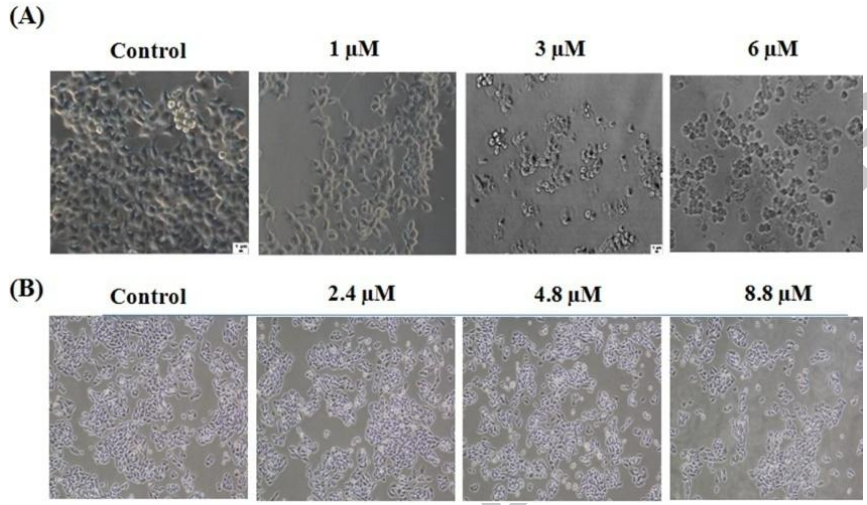



**Figure 4:** ***Morphological changes induce by compound 26 and 17 in MCF-7 and PC-3 cells respectively.*** (A) MCF-7 and (B) PC-3 cells treated with different concentration of **26** and **17** for 24 h and images were captured by using Nikon microscope at 10X magnification.

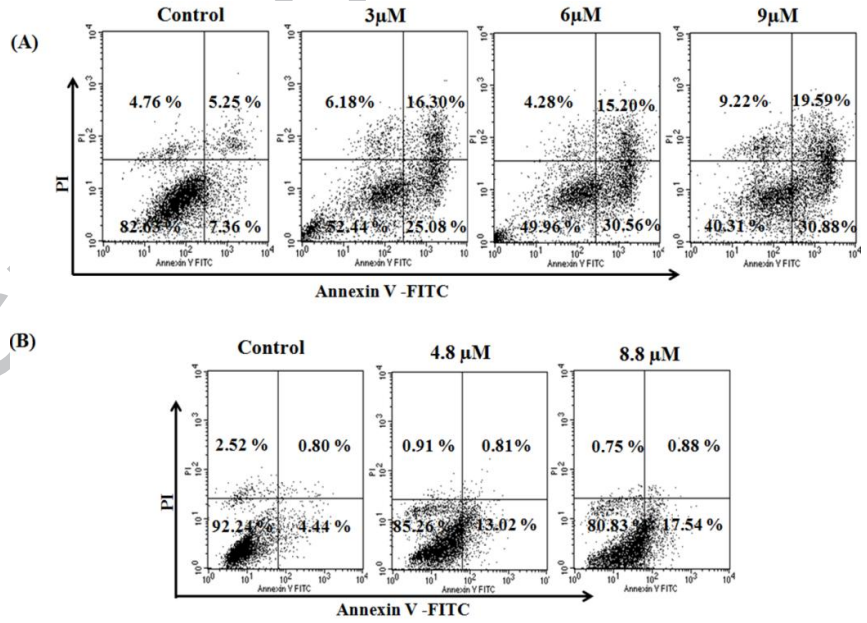



**Figure 5:** ***Compound 26 and 17 induces apoptosis in MCF-7 and PC-3 cells.*** (A) MCF-7 cells treated with compound **26** and (B) PC-3 cells were treated with **17** for 24 h. Cells were harvested, stained with Annexin V-FITC/PI and samples were acquired with flow-cytometer.

Curcumin conjugates were known to induce apoptosis in breast cancer cells via loss of mitochondrial membrane potential (MMP) [41]. Loss of MMP or mitochondrial depolarization is a common indication of involvement of intrinsic pathway of apoptosis. Therefore, cells were treated with different concentrations of most active compounds **17** and **26** for 24 h and alteration in MMP was measured by flowcytometry-based JC-1 staining method [43]. Compound **26** and **17** induced dose dependent increase of green population (suggestive of depolarized mitochondrial membrane) in MCF-7 and PC-3 cells respectively (Fig. 6). The increase of green population and decrease of red population (suggestive of polarized mitochondrial membrane) clearly indicated the involvement of mitochondria in compound **26** and **17** induced cell death in both cell lines.

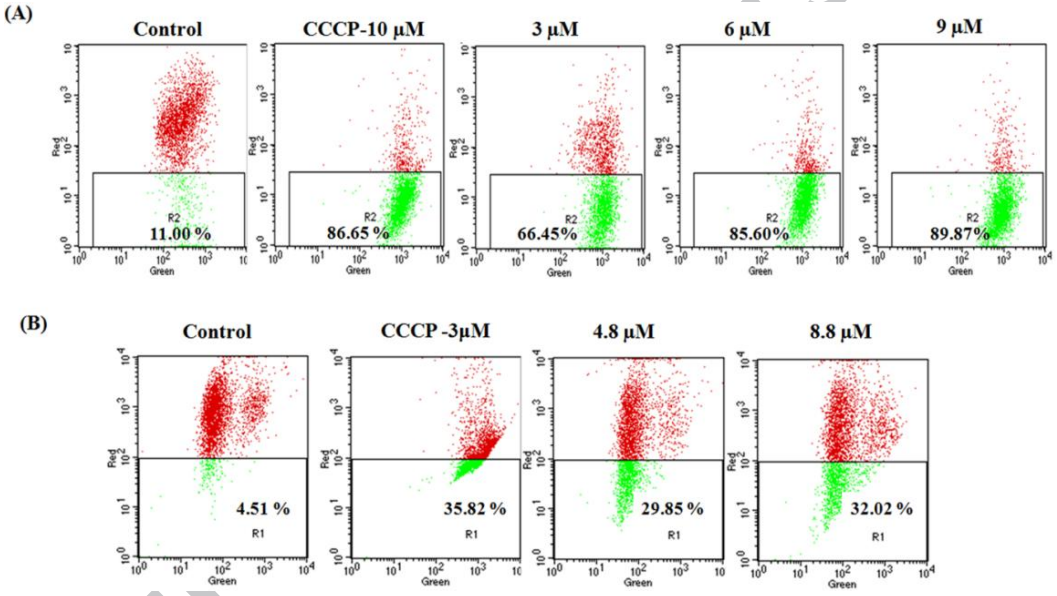



**Figure 6:** ***Effect of Compound 26 and 17 on mitochondrial membrane potential in MCF-7 and PC-3 cells.*** **(A)** MCF-7 cells treated with compound **26** (B) PC-3 cells were treated with Compound **17** for 24 h. Cells were harvested, stained with JC-1 and samples were acquired by flow cytometry.

In order to investigate the effect of compound **26** and **17** on cellular signaling pathways that regulate cell proliferation, survival and apoptosis, western blotting was carried. There was a decrease of proliferating cell nuclear antigen (PCNA), an important cell cycle regulatory protein in both the MCF-7 and PC-3 cells following compound treatment (Fig. 7). However, effect was more promising in case of PC-3 cells. Furthermore, increased expression of pro-apoptotic markers, Bax and decrease expression of anti-apoptotic markers, Bcl-2 was observed following compound treatment for 24 h in both cell lines (Fig. 7).

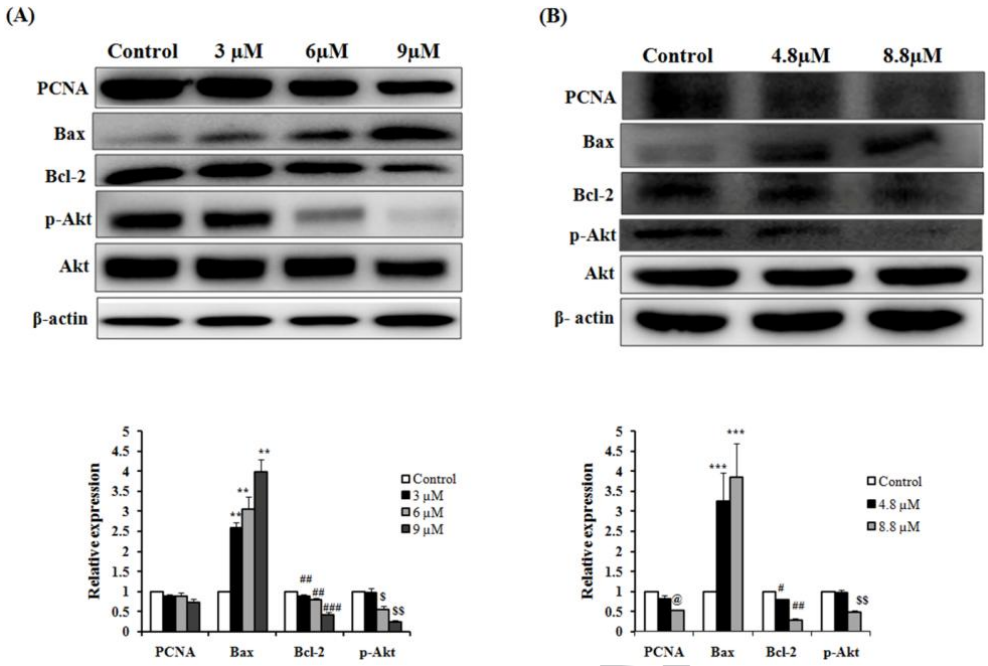



**Figure 7:** ***Effect of 26 and 17 on cellular signaling in MCF-7 and PC-3 cells.*** (A) MCF-7 cells were treated with compound **26** (B) PC-3 cells were treated with **17** for 24 h. Cells were harvested and protein expression was checked by Western blotting.

Among the signaling pathways implicated for endocrine cancers, activated Akt plays an important role in cell proliferation and it has been shown to be constitutively over expressed in cancers. It was widely reported that phytochemicals like, curcumin and other curcumin mimics down regulate the Akt phosphorylation [12, 42]. Phosphorylation of Akt, an indicator of cell survival was drastically inhibited in both the cell lines with treatment of compounds (Fig. 6). Compound **26** and **17** at concentration of 9 µM and 8.8 µM respectively, resulted in complete inhibition of Akt phosphorylation in respective MCF-7 and PC-3 cells (fig. 6). Collectively, these data suggest that compound **26** and **17** modulate cellular signaling of both breast cancer cells and prostate cancer cells resulting in inhibition of cell proliferation and induction of apoptosis.

In addition, *in vitro* stability and plasma binding of compounds **17** and **26** was assessed. In simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), compound **17** was 92% and 30% intact in SGF after 1 hr and SIF after 2 hr, respectively. The other compound **26** was 85% and 65% stable under the established experimental conditions after 1 hr and 2 hr respectively.

**Figure 8:**

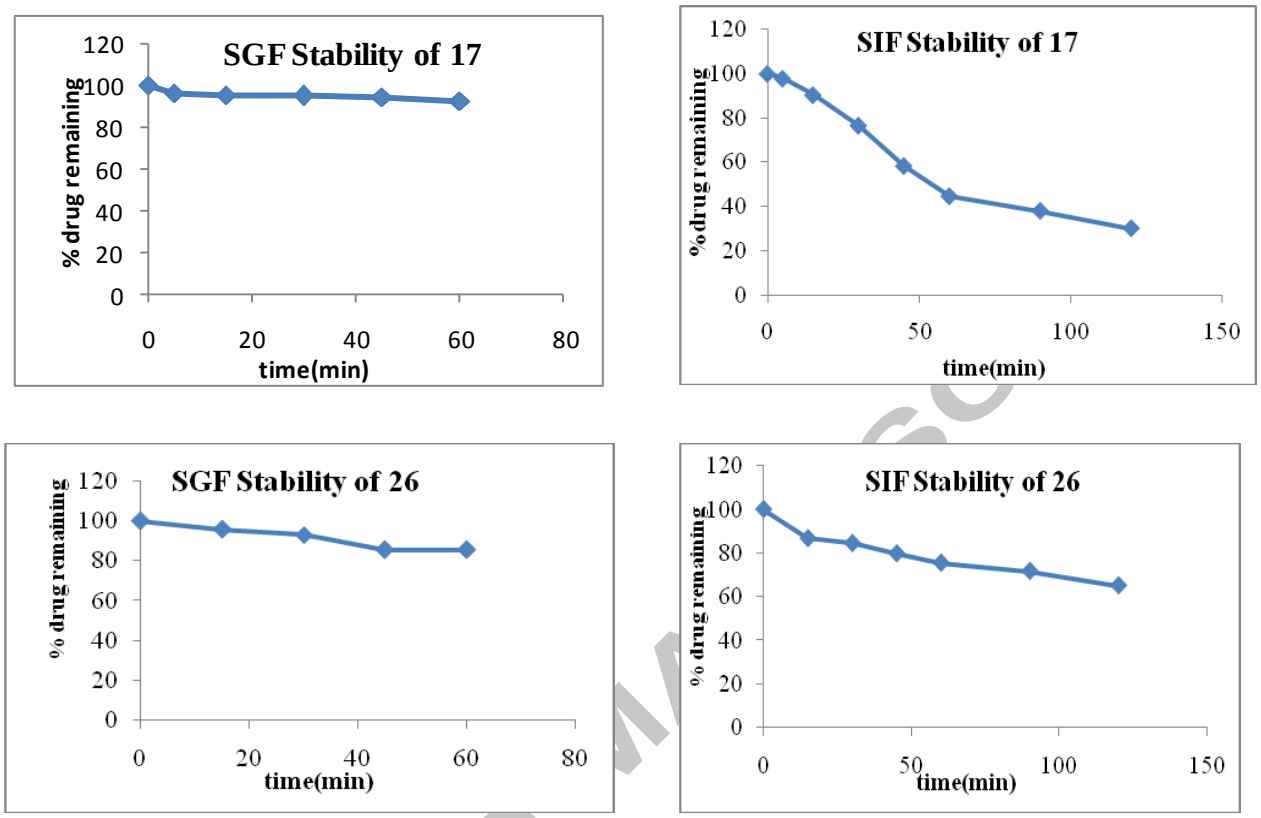



**Figure 8:** *In vitro* SGF and SIF stability of compounds **17** and **26**

In plasma stability study, the percentage of compound remaining intact at different time intervals after incubation was determined by the following formula.

$$\% \text{ Drug remaining} = \frac{\text{Concentration of drug after incubation}}{\text{Concentration of drug before incubation}} \times 100$$

Both the compounds were found to be stable in plasma as 90% of **17** and 96% compound **26** was intact after 2 hr of incubation.

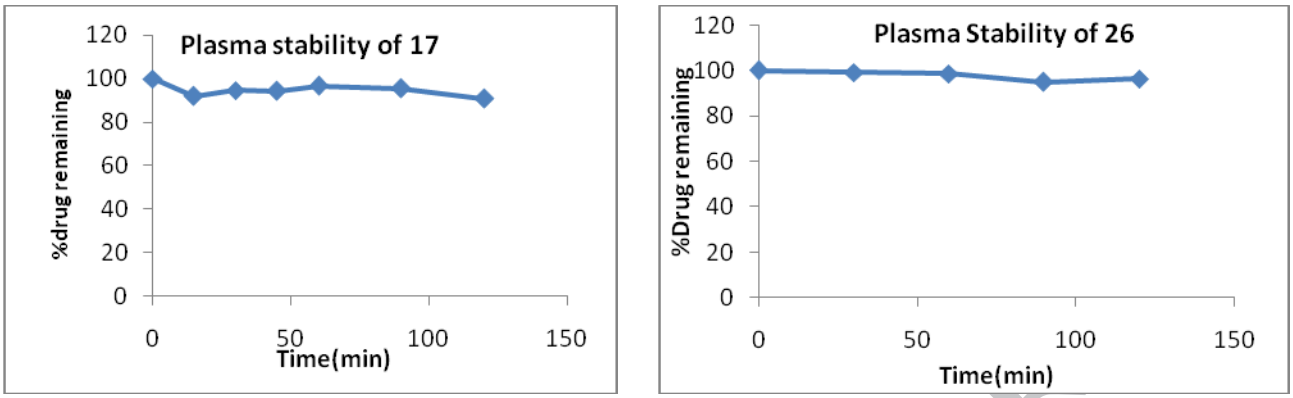



**Figure 9:** *In vitro* plasma stability of compounds **17** and **26**

In the *in vitro* metabolic study, the compound **17** was metabolised instantaneously as 66% compound was intact after 5 min in presence of microsomes where the other compound **26** found to be 46% intact after after 5 min. Whereas the parent compound (curcumin) was found to be metabolised instantaneously as only 45% was found (Table 2). Metabolic stability of curcumin-1,2,3-triazole conjugates were found to be ≥ to that of the curcumin in case of **17** and less in case of **26** based on the results obtained with curcumin as a control.

$$\% \text{ Drug remaining} = \frac{\text{Concentration of drug after incubation}}{\text{Concentration of drug before incubation}} \times 100$$

Elimination rate constant was obtained from natural log of % parent compound remained *versus* time plot, and the parameters estimated were as *in vitro* half-life, intrinsic clearance ($Cl_{int}$) intrinsic hepatic clearance ($Cl_{int.H}$), respectively. Following formulae were used:

$$t_{1/2} = \frac{0.693}{K\ (min^{-1})}$$

$$\text{Intrinsic Clearance } (CL_{int}) = k\ (min^{-1}) \times \frac{[V]\text{incubation (mL)}}{[P]\text{incubation(mg)}}$$

$$\text{Hepatic Clearance } (CL_{int,\,h}) = (CL_{int}) \times \left(\frac{\text{Protein (mg)}}{\text{Liver (g)}}\right) \times \left(\frac{\text{Liver(g)}}{\text{body weight(kg)}}\right)$$

**Table 2.** *In vitro* metabolic stability data of compounds **17**, **26** and curcumin.

| | Compound **17** | Compound **26** | **Curcumin** |
|---|---|---|---|
| K ($min^{-1}$) | 0.044 | 0.088 | 0.051 |
| Half-Life (min) | 15.89 | 7.52 | 13.68 |
| Intrinsic Clearance (mL/min×mg) | 0.087 | 0.176 | 0.101 |
| Hepatic Clearance (L/h/kg) | 157.52 | 316.8 | 182.39 |

In plasma protein binding study the percent protein binding (%) was estimated from the percent compound remaining in the supernatant (plasma) after adding the charcoal. Analysis was done using Ultra Performance Liquid Chromatography (LC-30AD) with photodiode array (SPD-M20A) detection system. The extent of protein binding of drug at time (t=0) was calculated and found to be 96.99% for **17** and 81.24% for **26**.

Further solubility of the compounds **17**, **26** and curcumin was found to be 1.12, 2.08 and 0.06 mg/L, respectively. Solubility of the compounds (**17** and **26**) was found to be 18 and 34 times higher than curcumin.

In conclusion, this study reports synthesis and biological evaluation of new monocarbonyl curcumin analogue-1,2,3-triazole conjugates. This series of hybrid compounds exhibited potent anti-cancer activity against endocrine related breast and prostate cancer. All the compounds inhibited proliferation either one or both type of the cancer cells. SAR revealed that incorporation of dithiocarbamate group (prototype-2) in the active monocarbonyl curcumin analogue-1,2,3-triazole scaffold (prototype 1) resulted in reduced activity. It was also found that among the more active compounds **17-28,** 2-fluoro and 4-methyl groups at $R^1$ position maintained good activity against breast and prostate cancer cells, respectively. Among the compounds synthesized, twelve compounds (**17-21**, **24-28**, **30** and **33**) were found to be active against both breast and prostate cancer cells. Compound **17** showed remarkable activity against prostate cancer cells with high safety index (>11) whereas compound **26** showed remarkable activity against breast cancer with good safety index (>7.6). The most active compounds (**17** and **26)** of the series induced cell cycle arrest, caused mitochondrial mediated apoptosis by modulating the expression levels of various cellular signaling proteins like Akt, PCNA Bax and

Bcl-2 in both breast and prostate cancer cells. In addition, *in vitro* stability, plasma binding and solubility studies showed that compound **17** and **26** were relatively stable.

In summary, we identified two novel monocarbonyl curcumin analogue-1,2,3-triazole conjugates, **17** and **26** with more potent activity than curcumin against endocrine-related cancers. Compounds **17** and **26** induced loss of cell viability, arrest of cell cycle progression and induced apoptosis by inhibiting Akt phosphorylation in human breast and prostate cancer cells, respectively. Our findings demonstrated that these two compounds could serve as potential lead molecules for the future development of novel cancer chemotherapeutic agents against breast and prostate cancers.

**Acknowledgement**

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**Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://..... (Synthetic procedures for compounds (**5-40**), scan copies of $^{1}$HNMR and $^{13}$C NMR spectral data of compounds **8-40**, HRMS spectra of **17-40** associated with this article and biological methods).

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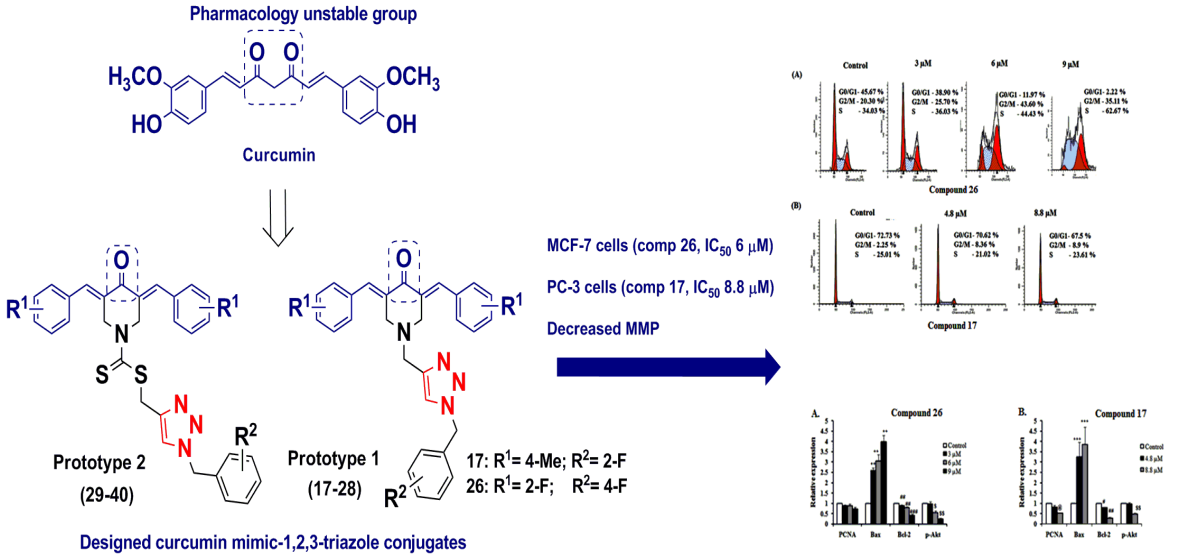
***Research Highlights:***

1. Curcumin mimic–1,2,3-triazole conjugates with two prototypes were synthesized.

2. Compounds **17** and **26** displayed significant anticancer activity than curcumin.

3. Compounds **17** and **26** induced mitochondrial-mediated apoptosis and cell cycle arrest.

4. Also significantly down regulated phospho-Akt, PCNA, Bcl-2 and upregulated Bax.




Designed curcumin mimic-1,2,3-triazole conjugates