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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

# Synthesis, characterization, antioxidant, and anticancer studies of 6-[3-(4-chlorophenyl)-1*H*-pyrazol-4-yl]-3-[(2-naphthyloxy) methyl][1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole in HepG2 cell lines

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**Abstract** Triazolo- thiadiazoles exhibit a variety of pharmacological properties, due to their cytotoxicity. In continuation of a previous study on triazolo-thiadiazoles, the authors have synthesized a new thiadiazole, 6-[3-(4-chlorophenyl)-1-*H*-pyrazol-4-yl]-3-[(2-naphthyloxy)methyl] [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (CPNT), which was further characterized by advanced spectral techniques and elemental analysis. The compound exhibited a dose-dependent cytotoxic effect on hepatocellular carcinoma cell line, HepG2 with very low $IC_{50}$ value of 0.8 μg/ml in 24 h when compared with standard drug, doxorubicin. Incorporation of [$^3$H] thymidine in conjunction with cell cycle analysis suggested that CPNT inhibited the growth of HepG2 cells. Flow cytometric studies revealed more percentage of cells in subG1 phase, indicating apoptosis, which was further confirmed through chromatin condensation studies by Hoechst staining. In vitro antioxidant activity of CPNT was determined by DPPH and ABTS free radical scavenging assays which revealed increasing scavenging activity with increasing concentration of the compound when compared with reference ascorbic acid.

**Keywords** CPNT · HepG2 cell lines · Cytotoxicity · Antioxidant

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## Introduction

Hepatocellular carcinoma is one of the most common cancers in the world and accounts for very high percentage of deaths annually (Courtney *et al.*, 2008). The majority of patients with liver cancer die within a year after diagnosis. Presently practiced treatment for the disease mainly includes surgery and chemotherapy, but the curative effects of the existing chemotherapeutic drugs are not good enough as they have several side effects. Therefore, searching for highly efficient antitumor drug remains a hot research area. It has been proposed recently that apoptosis might be an important and ubiquitous mode of cell death for cells treated with chemotherapeutic drugs.

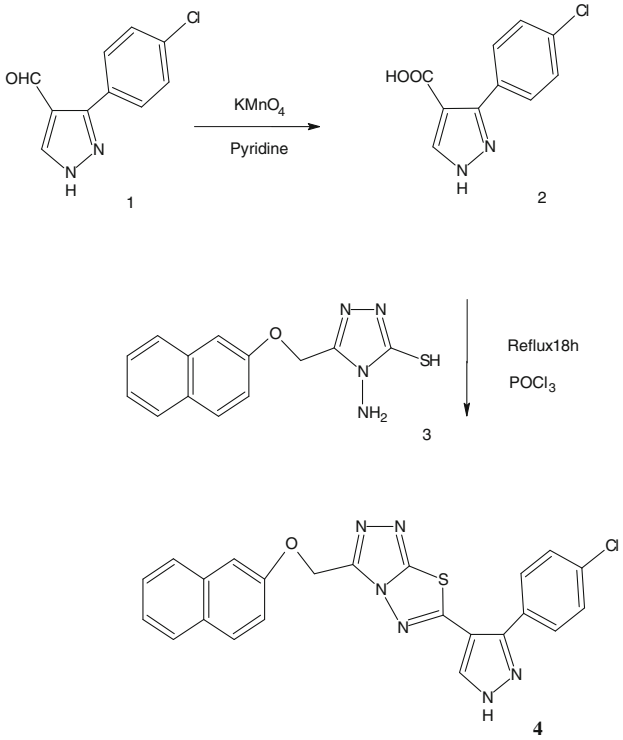
1,2,4-Triazole derivatives represent an interesting class of heterocyclic compounds with the advantage of broad spectrum activity, high oral availability, and toxicity (Shu-Sheng *et al.*, 2007). Triazoles and thiadiazoles have been reported to possess analgesic (Mathew *et al.*, 2007), anti-inflammatory (Mathew *et al.*, 2007, Kamotra *et al.*, 2007), antiviral (Tomita *et al.*, 2002), antimicrobia (Demirbas *et al.*, 2004, Swamy *et al.*, 2006), antifungal (Tsukuda *et al.*, 1998, Hirpara *et al.*, 2003, Isloor *et al.*, 2009), antibacterial (Wang *et al.*, 1996), antitubercular (Udupi *et al.*, 1999), and antitumor (Shivarama *et al.*, 2002) activities. Reports of compounds that contain two active groups, triazole and thiadiazole, in a single molecule have rarely been studied for its anticancer activity (Shu-Sheng

*et al.*, 2007). Also, in vitro and in vivo data suggest that certain antioxidants selectively inhibit the growth of tumor cells, may induce cellular differentiation, and may alter the intracellular redox state, thereby enhancing the effects of cytotoxic therapy (Lamson and Brignall, 1999, Conklin 2000, 2002). In continuation of an earlier study (Dhanya *et al.*, 2009) on the synthesis of triazolo-thiadiazoles, the authors report in this article the synthesis of 6-[3-(4-chloro-phenyl)-1-*H*-pyrazol-4-yl]-3-[(2-naphthyloxy)methyl][1,2,4] triazolo[3,4-*b*][1,3,4] thiadiazole (CPNT) and its dose-dependant anti-cell proliferative efficiency leading to apoptosis in HepG2 cell lines. The high antioxidant capacity of CPNT was confirmed by 1,1-diphenyl-2-picryl hydrazide (DPPH) and 2,2-azino bis 3-ethyl benzo-thiazoline-6-sulphonic acid (ABTS) assays.

## Results and discussion

### Synthesis

3-Methyl-$\beta$-naphthyloxy-4-amino-5-mercapto-1,2,4-triazole **3** was synthesized as per the method in the literature (Isloor *et al.*, 2000). 3-(4-Chloro phenyl)pyrazole aldehyde **1** was prepared as reported in the literature (Kalluraya *et al.*, 2001, 2004, Singh *et al.*, 2005). The aldehyde obtained was converted into pyrazole acid **2** by oxidation using potassium permanganate in pyridine medium (Bratenko *et al.*, 2001). Subsequently, 3-methyl-$\beta$-naphthyloxy-4-amino-5-mercapto-1,2,4-triazole **3** and 3-(4-chloro phenyl)pyrazole acid **2** were refluxed for 18 h in phosphorous oxychloride medium which resulted in cyclized title compound **4**. The reaction sequence is outlined in Scheme 1.




**Scheme 1** Synthetic route for triazolothiadiazole

### Characterization

The characterization of CPNT was done using advanced spectroscopic studies and elemental analysis. Melting point was determined by open capillary method. Thin layer chromatography was conducted on $0.25 \times 10^{-3}$ m silica gel plates to monitor the progress of the reaction and to check the purity of the compound. A 1:1 mixture of ethyl acetate and petroleum ether solution was used as the eluent. Visualization was made by using iodine vapors. The IR spectrum in KBr pellets was recorded on a Schimadzu FTIR 8400S spectrophotometer. $^1$H-NMR spectrum was recorded in deuterated dimethyl sulphoxide on an AV500 NMR spectrometer using TMS as an internal standard. The mass spectrum was recorded on a Schimadzu GCMS-QP5050 mass spectrometer. The elemental analysis was done using Flash thermo 1112 series CHN analyzer.

### Anticancer studies

Unless otherwise mentioned, all the chemicals used in this study were from Sigma-Aldrich, USA. The hepatocellular carcinoma cell line, HepG2, was used for all the analyses in this study. HepG2 cell lines were purchased from National Center for Cell Science, Pune, India. The cell line was cultured in DMEM containing 10% FBS at 37°C in an atmosphere containing 5% $CO_2$.

#### *MTT assay*

Cytotoxic effect of CPNT was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann 1983, Kishore *et al.*, 2008). Cells were seeded in duplicates in 96-well plates at $1 \times 10^4$ cells/well. After 24 h, CPNT was added at a concentration of 0.1, 1, 10, and 100 µg/ml and incubated for another 24 h. 5 mmol of MTT reagent was added and incubated for additional 4 h. The purple formazan crystals were dissolved in 0.1 ml of hydrochloric acid (0.4 N): isopropanol (1:24). Cells grown in culture media alone and with appropriate concentration of DMSO were used as control and vehicle control, respectively. Doxorubicin was used as the standard drug. The optical density of each well was measured at 570 nm in an ELISA plate reader. The results were reported as percentage survival of the cells in

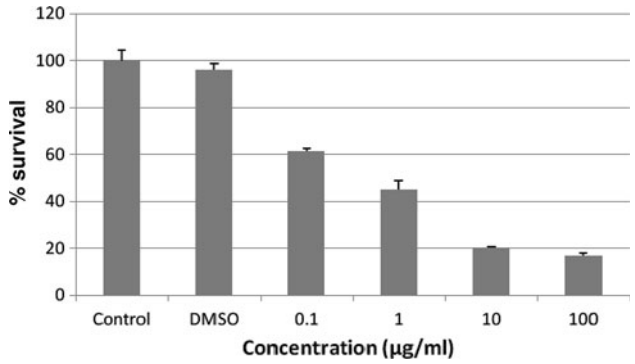



**Fig. 1** Cytotoxic analysis of CPNT in HepG2 cell lines using MTT assay. DMSO acts as vehicle control. In case of control, neither CPNT nor DMSO was added. Cells were treated with 0.1, 1, 10, and 100 µg/ml of CPNT. The percentage viability was calculated considering control wells as 100% and plotted with representation of *error bars*

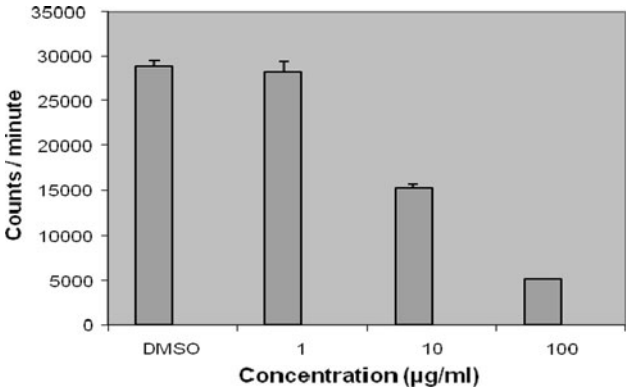



**Fig. 2** [$^3$H] thymidine incorporation assay to determine effect of CPNT on cell proliferation. The data presented are the results of duplicates, and *error bars* are indicated

comparison to that of the untreated control cells ± standard deviation (Fig. 1). The $IC_{50}$ of CPNT was found to be 0.8 µg/ml which was significantly low when compared to that of the standard drug, doxorubicin.

### *[$^3$H] Thymidine incorporation assay*

Since the above MTT assay suggested that CPNT affects the cell viability, we were interested in knowing whether it could inhibit cell division. DNA synthesis was monitored by labeling cells using [$^3$H] thymidine (Kavitha *et al.*, 2009). Around $1 \times 10^6$ cells/ml were seeded in duplicates, CPNT was added at a concentration of 1, 10, and 100 µg/ml and incubated for 3 h. $18.5 \times 10^3$ Bq of [$^3$H] thymidine was added and incubated for additional 2 h. The cells were collected by Millipore filtration, washed with cold 10% trichloro acetic acid (TCA), methanol, and water. Filters were removed into liquid scintillation vials, dried, cocktail added. and radioactivity was measured using a liquid scintillation beta counter. Radioactivity was expressed as cpm (counts per minute), which was proportional to the amount of [$^3$H] thymidine incorporated into the DNA of cultured cells ± standard deviation. CPNT treatment showed a dose-dependant reduced incorporation of [$^3$H] thymidine drastically, suggesting that it affects the cell viability by inhibiting cell division probably by interfering with DNA replication (Fig. 2). However, it is also possible that in addition to its effect on cell division, CPNT could induce apoptosis.

### *Cell cycle analysis (Kavitha et al., 2009, Ormerod et al., 1994)*

Hep G2 cells were cultured and treated with two concentrations, 0.06 and 0.08 µg/ml of CPNT, in duplicates for 24 h to study concentration-dependent effect on the cell cycle arrest. Cells were fixed in 2 ml of 70% alcohol, centrifuged at 3000 rpm for 10 min, and alcohol was discarded. The cells were washed in 2 ml PBS, and to the pellet, 0.015 ml of RNase was added, followed by 0.01 ml of propidium iodide. The cells were incubated for 1 h and subjected to flow cytometry. The results were analyzed using cell quest pro software using excitation at 488 nm laser and emission at 560/670 nm. A minimum of 10,000 cells were acquired and histograms analyzed. The total number of cells included in analysis was taken as 100%. The histogram of DMSO-treated cells showed a standard cell cycle pattern, which includes G1 and G2/M peaks separated by S phase peak. The subG1 peak showed very less percentage of dead cells. Interestingly after addition of CPNT, a concentration-dependent change was observed in the percentage of cells in each phase of the cell cycle. There was a remarkable dose-dependent increase in the percentage of sub-ploid cells in the subG1 phase. We could also observe more cells in the G1 phase when compared to S and G2phases indicating a cell cycle arrest probably in the G1 phase of the cell cycle allowing fewer cells to enter into the S phase confirming the results obtained in the [$^3$H] thymidine incorporation assay. The bar diagram quantifying the percentage of cells in the different phases of the cell cycle in control and treated with different concentrations of CPNT is shown in Fig. 3. The flow cytometry data are represented in Table 1.

### Chromatin condensation studies (Hoechst staining)

To confirm the action of CPNT through growth inhibition mediated by a DNA replication defect followed by apoptosis, chromatin condensation studies were conducted. Condensation of chromatin is usually the late event in apoptosis. HepG2 cells were grown in 96-well plates in duplicates and subjected to the treatment of CPNT in two different concentrations of 10 and 100 µg/ml and incubated for 24 h. About 0.05 ml of the medium was aspirated, the

same amount of diluted Hoechst dye was added and incubated for 5 min. 0.05 ml of the medium was removed and viewed under fluorescence microscope. Hoechst dye binds at the adenine–thymine-rich regions of DNA and emits blue fluorescence when excited by UV light about 350 nm. DMSO-treated wells were used as control (Fig. 4a). The presence of dose-dependent increase in condensed apoptotic chromatin confirms the apoptotic action of CPNT (Fig. 4b, c).

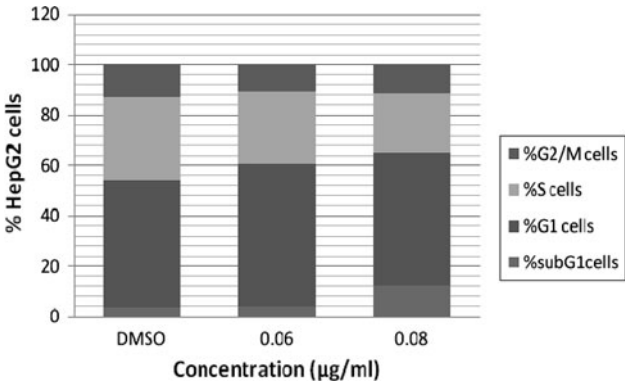



**Fig. 3** Bar diagram showing quantification of cells in each phase of the cell cycle in flow cytometry studies

## Antioxidant studies

### *DPPH radical scavenging assay (Sreejayan and Rao, 1996, John and Steven, 1984)*

The DPPH antioxidant assay is based on the ability of DPPH, a stable, free radical, to decolorize in the presence of antioxidants. DPPH radical scavenging is mediated by transfer of hydrogen atom. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. 100 µl of various concentrations of CPNT was added to respective wells of a 96-well micro-plate. Equal amount of DPPH (1,1-diphenyl-2-picryl hydrazide) was also added to each well to make up a final volume of 200 µl. After 20-min incubation in the dark, the ability of CPNT to scavenge the free radical DPPH was measured by recording the absorbance at 517 nm using an ELISA plate recorder. Experiment was

**Table 1** % cells in different stages of the cell cycle—flow cytometric analysis

| Concentration | $M_1$ (%subG1 cells) | $M_2$ (%G1 cells) | $M_3$ (%S cells) | $M_4$ (%G2/M cells) |
|---|---|---|---|---|
| Control (DMSO) | 3.57 ± 0.03 | 50.68 ± 1.42 | 32.84 ± 1.06 | 12.92 ± 0.27 |
| 0.06 µg/ml | 4.00 ± 0.06 | 56.59 ± 1.96 | 28.82 ± 0.98 | 10.58 ± 0.51 |
| 0.08 µg/ml | 12.33 ± 0.09 | 52.65 ± 1.37 | 23.68 ± 0.67 | 11.33 ± 0.24 |

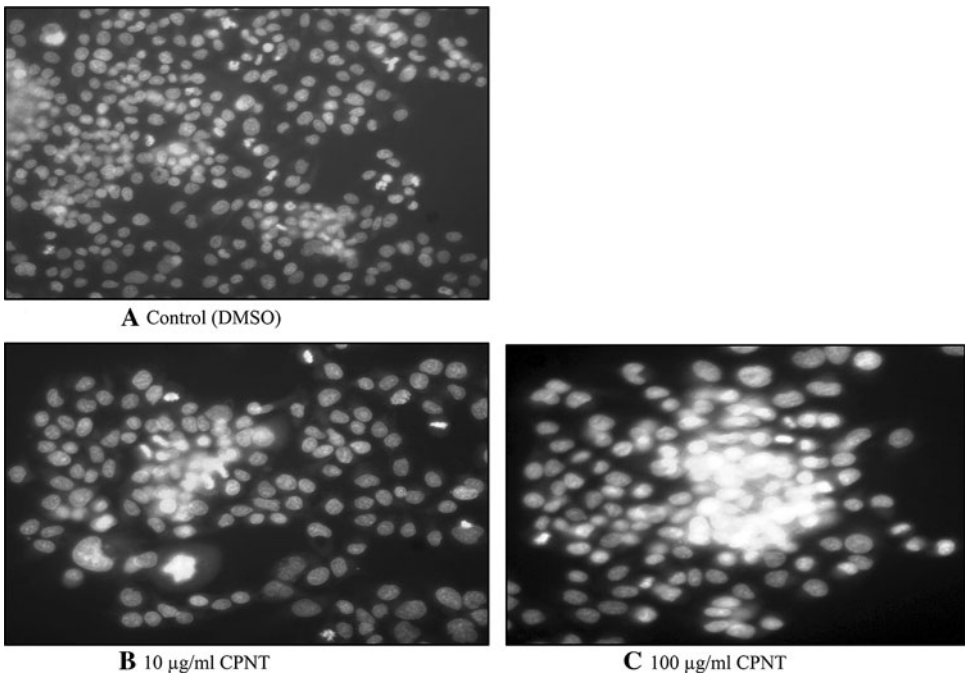



**Fig. 4** Chromatin condensation studies (Hoechst test). Dose-dependent increase in condensed apoptotic chromatin is visible on treatment with CPNT when compared to control

performed in triplicates, and average values were considered. An equal amount of each of methanol and DPPH was added to the control. Ascorbic acid was used as reference standard. Comparison between the antioxidant activity of CPNT and ascorbic acid is shown in Fig. 5.

$$\%\,\text{Scavenging of DPPH} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

The $IC_{50}$ values for CPNT and ascorbic acid for scavenging DPPH were found to be 1.023 μg/ml and 1.46 μg/ml, respectively. CPNT was found to be more potent than ascorbic acid.

*ABTS radical scavenging assay (Vaijanathappa et al., 2008)*

ABTS is chemically 2,2-azino bis 3-ethyl benzo-thiazoline-6-sulphonic acid. The reduction of this radical by CPNT is measured at 690 nm. The electron transfer capability of CPNT was studied using ABTS radical scavenging assay. In a 96-welled microtiter plate, 40 μl of the CPNT/ascorbic acid, 200 μl of methanol, and 30 ul of ABTS solution were added. The plate was then incubated at 37°C for 20 min after which the absorbance was measured at 690 nm using an ELISA plate reader. Sample blank and control were also taken. The experiment was performed in triplicates, and average values were considered. The comparative values of the antioxidant activity of CPNT and ascorbic acid is depicted in Fig. 6.

$$\%\ \text{Scavenging of ABTS} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

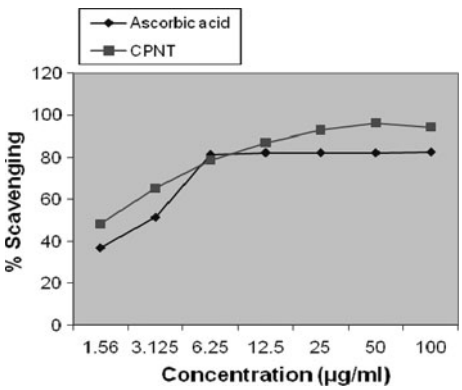



**Fig. 5** DPPH radical scavenging activity of CPNT added to methanol solution of DPPH. Radical scavenging activity was measured at 517 nm as compared to standard ascorbic acid. Values are the average of triplicate experiments

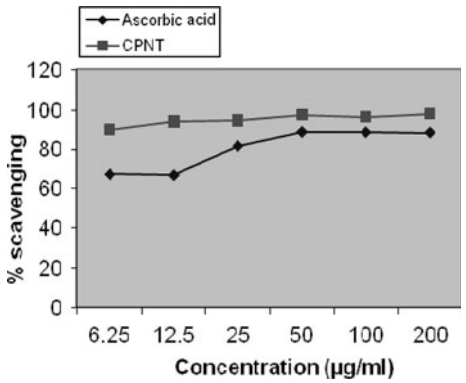



**Fig. 6** ABTS radical scavenging activity of CPNT measured at 734 nm as compared to standard ascorbic acid. Values are the average of triplicate experiments

CPNT was found to be more potent when compared with ascorbic acid in ABTS assay.

## Conclusion

In this study, a new triazolo thiadiazole CPNT was synthesized, which showed a dose-dependent cytotoxic effect, the $IC_{50}$ being 0.8 μg/ml which is found to be significantly low when compared to standard drug, doxorubicin with $IC_{50}$ 19 μg/ml. Treatment of HepG2 cells with CPNT showed a dose-dependent decreased cell division in [$^3$H] thymidine incorporation assay and increase in the subG1 population in flow cytometric analysis. The chromatin condensation studies confirmed the apoptotic action of CPNT. The significant antioxidant activity of CPNT with low $IC_{50}$ values when compared with standard ascorbic acid is clearly evident from DPPH and ABTS free radical scavenging assays. As ABTS scavenging occurred at a lower concentration than DPPH scavenging, it may be presumed that the antioxidant action of CPNT is primarily through the mechanism of electron transfer rather than hydrogen transfer.

The ability to cross the biomembranes and bioavailability of CPNT is dependent on the lipophilic moiety attached. The title compound has naphthyloxy methyl and pyrazole substituents. The chlorine atoms also might have added to the potency of the compound as a chlorine substituent produces simultaneously an increase in lipophilicity, an electron-attracting effect, and a metabolic obstruction. In CPNT, since the bulky chlorine atom is present at the para position, it may provide less steric hindrance and could increase the binding affinity of the molecule. As this study reveals CPNT as a potent cytotoxic compound with low $IC_{50}$ values compared to the standard drug doxorubicin, leading to apoptotic pathway along with significant antioxidant activity, there is ample scope of further study in this area.

## Experimental

Preparation of 6-[3-(4-chlorophenyl)-1H-pyrazol-4-yl]-3-[(2-naphthyloxy)methyl][1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (4)

To a mixture of 2.72 g, 10 mmol of 3-methyl-$\beta$-naphthyloxy-4-amino-5-mercapto-1,2,4-triazole **3** and 2.22 g, 10 mmol of 3-(4-chloro phenyl) pyrazole 4-carboxylic acid **2**, and 20 ml of phosphorous oxychloride were added, and the contents were heated under reflux for 18 h. Excess phosphorous oxychloride was distilled off, and the residue was poured onto crushed ice with vigorous stirring. The resulting solid **4** was washed with cold water, 20% sodium hydrogen carbonate solution, and recrystallized from a mixture of ethanol and dioxane (1:1 mixture).

Characterization data of 6-[3-(4-chlorophenyl)-1H-pyrazol-4-yl]-3-[(2- naphthyloxy)methyl][1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (4)

Yield: 78%, m.p.; 150-152°C, IR (KBr) [$cm^{-1}$]; 1620 (C=N str.), 3090(Ar–H str.), 1495, 1380 (Ar.C=C str.), 820 (Ar.C–H def.), 750, 820, 840(naphthalene C–H), 1050, 1280 (Ar.C–O–C str.), $^{1}$H NMR (deuterated DMSO) [ppm]; 5.5 (2H, O–CH2), 6.9 (1H, pyrazole C–H), 7.1 (1H, pyrazole N–H), 7.5-8 (11Ar–H), $^{13}$C–NMR : 174.1, 167.1, 162.4, 157.2, 145.2, 136.1, 131.1, 129.4, 110.0, 105.9, MS(m/z); 458($M^+$), 460 (M+2), 315 ($M^+$ of 6- [3-(4-chlorophenyl)-1 *H*-pyrazol-4-yl]-3-methyl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole), 317 (M+2 of 6-[3-(4-chlorophenyl)-1*H*-pyrazol-4-yl]-3-methyl [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole), 144 ($M^+$ of 2-naphthol), 280 ($M^+$ of 3-methyl-6-(3-phenyl-1*H*pyrazol-4-yl)[1,2,4] triazolo[3,4-*b*][1,3,4]thiadiazole), elemental analysis calcd. (%) for $C_{23}H_{15}N_6OClS$: C, 60.19; H, 3.27; N, 18.32. Found (%): C, 60.06; H, 3.23; N, 18.34.

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