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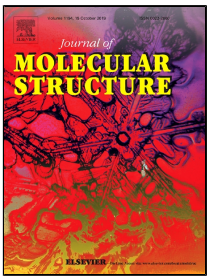

Synthesis, characterisation of new derivatives with mono ring system of 1,2,4-triazole scaffold and their anticancer activities

Mukhlif Mohsin Slaihim, Fouad Saleih R. Al-Suede, Melati Khairuddean, Mohamed B. Khadeer Ahamed, Amin Malik Shah Abdul Majid

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SYNTHESIS, CHARACTERISATION OF NEW DERIVATIVES WITH MONO RING SYSTEM OF 1,2,4-TRIAZOLE SCAFFOLD AND THEIR ANTICANCER ACTIVITIES

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ABSTRACT

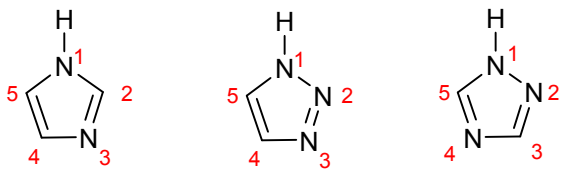
In the present study, two important starting materials and 18 new 1,2,4-triazole compounds with mono ring system have been synthesized and characterized. The mono system showed 16 compounds of a Schiff base moiety attached to the triazole ring which was prepared from the corresponding starting material **5(A-B)** or piperidinium salt system **6(A-B)**. All these compounds were characterized using Fourier Transform Infrared (FT-IR) and Nuclear Magnetic Resonance (NMR) spectroscopy and carbon hydrogen nitrogen (CHN) elemental analysis. The compounds were selected for *in vitro* anticancer study to test the therapeutic cytotoxic potential against cancer cells. The MTT test was conducted against human breast (MCF-7) and colorectal (HCT-116) cancer cells. Among all the compounds tested, **7A-i** demonstrated more pronounced *in vitro* anticancer effect against MCF-7 and HCT-116 cells with IC_{50} of 38 and 19.2 μM, respectively, comparable to that of the standard reference drugs, tamoxifen and 5-fluorouracil, respectively. Compound **7A-vi** showed a considerable cytotoxic effect with IC_{50} 53 and 41.2 μM against MCF-7 and HCT-116 cells, respectively. Compounds **7A-ii, 7A-iii** and **7A-v** exhibited moderate cytotoxicity with IC_{50} 68, 91 and 85 μM, respectively against MCF-7 cells and also 59.3, 81.7 and 137.1 μM against HCT-116 cells, respectively. However, all other compounds tested in this study showed poor cytotoxicity against both the cell lines. Cellular morphological analysis revealed that the cytotoxicity induced by the compounds could probably due to autophagy. It can be concluded that 1,2,4-triazole derivatives can be promising therapeutic agents. Further studies will be done to investigate the antitumor efficacy of the 1,2,4-triazole derivatives using suitable preclinical models.

Keywords:1,2,4-triazole, pyridinium salt, piperidinium salt, anticancer activity, MTT assay, anti-proliferation.

1. Introduction

The discovery and development of new effective and selective anticancer drugs are of high importance in modern cancer researches. Cancer is a major worldwide problem which is characterized by proliferation and spreading of abnormal cells uncontrollably and causes the cells to eventually undergo structural changes to become malignant [1]. Treatment of cancer by chemotherapy showed limitation due to the negative side effects. Since chemotherapeutic drugs cannot differentiate between the cancer cells and normal cells, these drugs often became toxic to human vital organs such as heart and kidney [2]. The emergence of chemotherapeutic drug resistance in cancer cells also resulted in tumor recurrence [3,4]. Today, the discovery and development of effective anticancer agents are the key focus of most researchers in medicinal chemistry. In spite of a large number of chemotherapeutic drugs available for medical usage, the increasing resistance made it necessary to continue the search for new potential anticancer drugs.

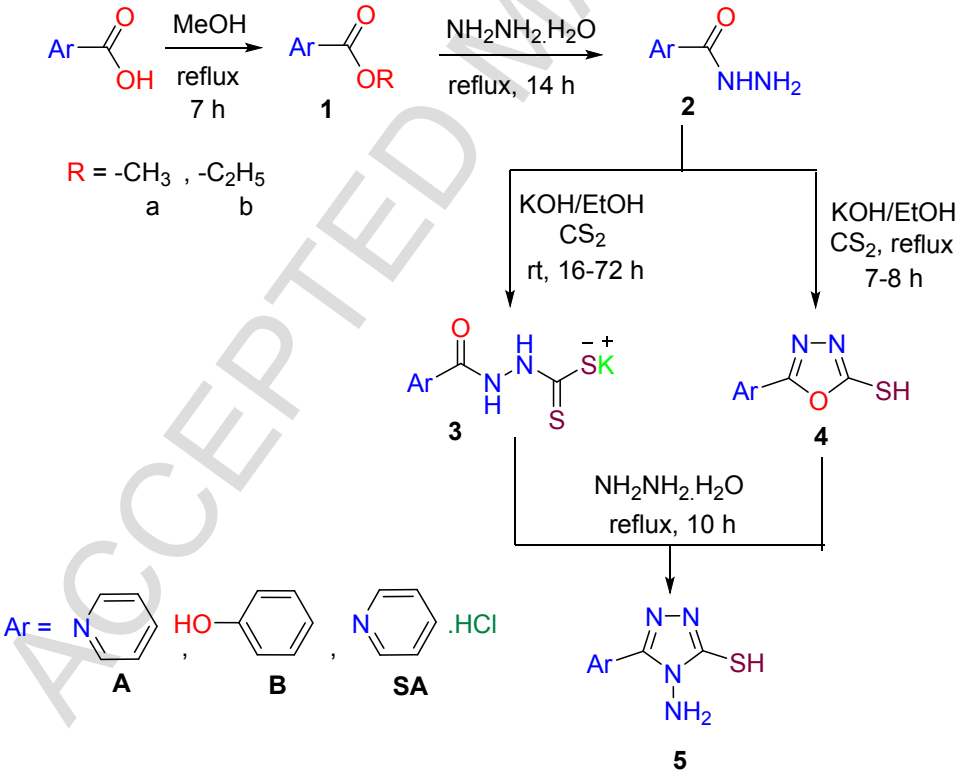
In the field of pharmaceutical organic chemistry, research is concentrated towards the introduction of new and safe therapeutic agents of clinical importance. Recently, 5-membered heterocyclic compounds have proved their importance as being the center of the biological activities [5,6]. The nitrogen-containing heterocycles are found in abundance in most of the medicinal compounds. Starting with imidazole as an important moiety of the medicinal agent, research work has led to the introduction of triazole which is an isostere of imidazole, in which one of the carbon atoms of imidazole is replaced by nitrogen [7-9].



The discovery of 1,2,4-triazole ring represents an interesting class of compounds with promising biological activities such as antibacterial [10,11], antimicrobial [12,13], anticonvulsant [14], antifungal [15], antidepressant [16] and anti-tuberculosis [17,18]. However, these types of derivatives, targeting specifically on the anticancer activity have not been widely explored [19-24]. This research work focused on the modification of 1,2,4-triazole scaffold into various bioactive structures and their subsequent evaluation for anticancer activities, focusing on the human breast (MCF-7) and colorectal (HCT-116) tumor cells. New findings have established the fact that fused 1,2,4-triazole ring contributed great significance in the field of medicinal chemistry due to their versatile biological properties [14].

2. Results and discussion

2.1 Spectral confirmation of the synthesized compounds



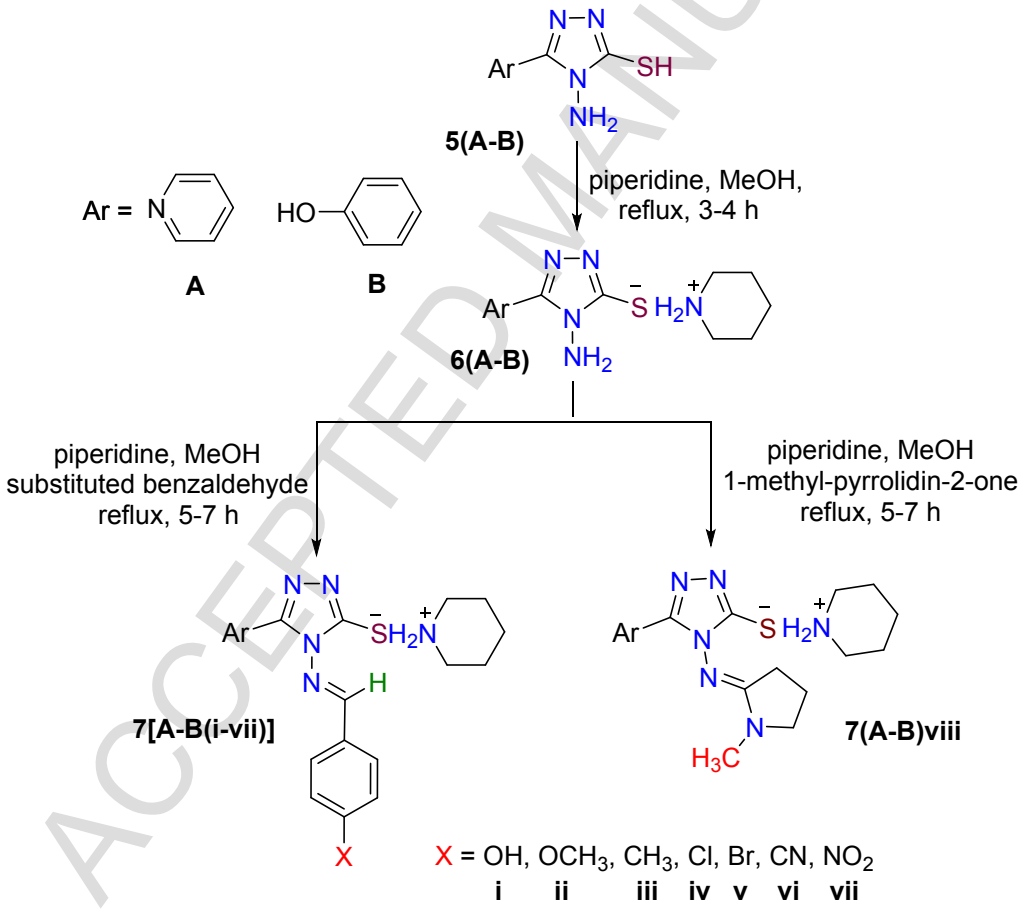
Scheme 1: Synthesis pathways of 1,2,4-triazole derivatives

In the series of esters, **1**, all the spectral data for methyl and ethyl 4-substituted-benzoate derivatives featured the main absorption peaks at 3078 cm^{-1} (C_{sp2}-H aromatic stretch), 2958 and 2855 (asymmetrical and symmetrical C_{sp3}-H stretch), 1716 (C=O stretch), 1608 and 1520 cm^{-1} (C=C aromatic stretch). Compounds with hydroxyl substituent showed the bands at 3369 cm^{-1} which corresponded to the O-H stretching. Since the hydroxyl groups are powerful *ortho*- and *para*- activators, the accretions of electron density in these positions led to the shielding effect which resulted in the upfield shifts of the NMR signal.

In the condensation reaction, carboxylic acid was transformed into the corresponding acid hydrazide, **2**. The absorption band in the IR spectrum showed the disappearance of the absorption band (O-H stretch) of the carboxylic acid and the appearance of the absorption band (N-H stretch) of the amide group. The ^{1}H-NMR spectrum revealed the presence of two doublets of the aromatic protons and two amino protons at δ 4.70 ppm (-NH_2) and 10.14 ppm (-NH). The reaction of acid hydrazide, **2** with CS_2 at room temperature yielded potassium dithiocarbazinate salts, **3**, the intermediates used to prepare 1,2,4-triazole derivatives. However, under reflux, the mixture gave 5-substituted-1,3,4-oxadiazole-2-thiol, **4**, instead, which were also used to prepare the corresponding 1,2,4-triazole derivatives (Scheme 1). In the IR spectrum, the disappearance of the strong absorption of C=O in acid hydrazide and the appearance of new bands of C=N in the range of 1604-1653 cm^{-1} confirmed the formation of 5-substituted-1,3,4-oxadiazole-2-thiol, **4**. The ^{1}H-NMR spectrum revealed the presence of the aromatic protons as two doublets at δ 6.94 and 7.72 ppm and two broad singlets at δ 10.42 and 14.54 ppm, assigned for protons of -OH and -SH, respectively. In the ^{13}C-NMR spectrum, eight carbon signals were observed. Intermediates **3** and **4** were refluxed separately with hydrazine hydrate to yield 1,2,4-triazole derivatives of salt **5SA** and 4-hydroxyphenyl derivatives of **5B**, respectively. The IR spectrum of **5SA** showed the appearance of a band at 1605 cm^{-1} (C=N stretch of the ring) and 2400 cm^{-1} (-$^{+}$N-H, a triazole compound existed in the

salt form). The ^{1}H-NMR spectrum showed two singlets at δ 14.18 (-SH) and 5.87 (-NH_2) ppm and two doublets for two aromatic protons at δ 8.76 and 8.03 ppm while the ^{13}C-NMR spectrum showed seven carbon signals whereby five signals were assigned to the pyridyl group and another two signals for carbons in the triazole ring [25].

A series of 1,2,4-triazole derivatives with Schiff base moiety were synthesized according to two new procedures (Scheme 2). In the first procedure, piperidine was added with intermediate **5A** which underwent the dehydrohalogenation to produce the cyclic imine [26]. In the second procedure, a mixture of **5A** in absolute methanol with a few drops of piperidine was refluxed before the addition of various aromatic aldehydes.



Scheme 2: Synthesis pathway of 1,2,4-triazole derivatives with Schiff base moiety

The IR spectrum of **6A** showed the absorption bands at 3418 (N-H stretch of piperidine); 3271 and 3160 (N-H stretch of triazole); 3058 (C_{sp2}-H stretch); 1605 (C=N stretch); 1571 and 1478 (C=C stretch); 1413 (C–N); 1217, 1085 and 1003 (C–N–C); 937, 823 and 687 cm^{-1} (1,2,4-triazole ring). The ^{1}H-NMR spectrum of **6A** revealed three different protons. The methylene protons showed three different signals. A signal at δ 2.99 ppm was integrated to four methylene protons of the piperidinium ring while two multiplets at δ 1.64 and 1.56 ppm were assigned for six methylene protons. The second type was the aromatic protons which featured two doublets at δ 8.64 and 8.03 ppm, integrated to four protons of the pyridyl group while the third type were the protons of the amine and ammonium groups which showed a singlet at δ 5.71 and 5.31 ppm, respectively. The ^{13}C-NMR spectrum of **6A** showed eight signals which represented all the 12 carbons. Signals at δ 149.8, 120.2 and 134.8 ppm were assigned to five carbons of the pyridyl group while signals at δ 168.4 and 146.2 ppm were assigned to two carbons of the triazole ring and signals at δ 22.1 to 44.3 ppm were for carbons of the piperidinium moiety of **6A**. The DEPT-135 NMR spectrum confirmed the methine and quaternary carbons in compound **6A**. The 2D-NMR correlation (^{1}H-^{1}H COSY and ^{1}H-^{13}C HMQC spectra) confirmed the assignment of the aromatic signals, especially the triazole protons. Further confirmation of **6A** provided by ^{1}H-^{13}C HMBC showed the short-range correlations observed between the carbon atoms of the aryl/phenyl.

For 1,2,4-triazole Schiff base derivatives (**7Ai-viii**), compound **7Ai** was used as a representative. The IR spectrum of **7Ai** (Figure 1) indicated the absence of the -NH_2 group but the appearance of the bands at 3331 (O-H stretch) and 1651 cm^{-1} (C=N stretch) which confirmed the formation of the Schiff base in **7Ai**. Other absorption bands were observed at 3001 (C_{sp2}-H stretch), 2955 (C_{sp3}-H stretch), 1592 and 1514 (C=C stretch) and 710 cm^{-1} (C-S).

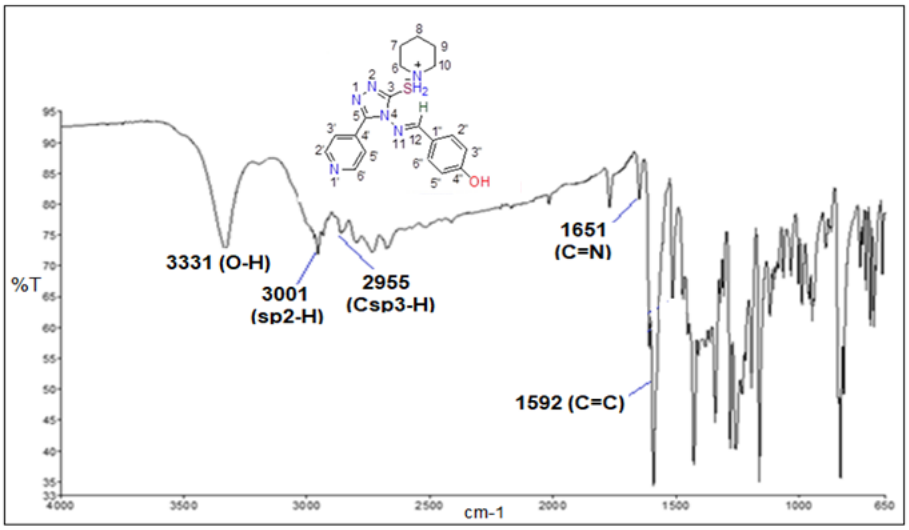


Figure 1: IR spectrum of **7Ai**

The ^{1}H NMR spectrum (Figure 2) of **7Ai** revealed signals of nine aromatic protons and five piperidine protons. The ^{13}C-NMR spectrum (Figure 3) of **7Ai** displayed signals of 14 aromatic carbons and five piperidine carbons.

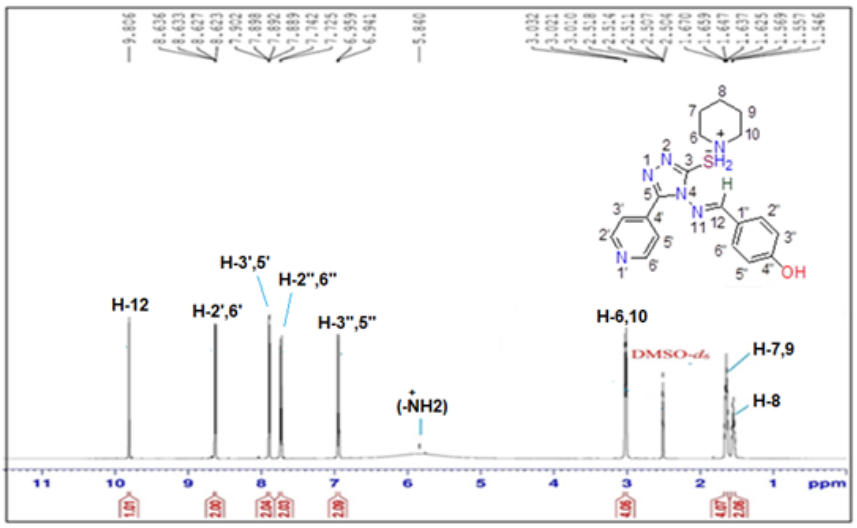


Figure 2: ^{1}H-NMR spectrum of **7Ai** (500 MHz, DMSO-d_6)

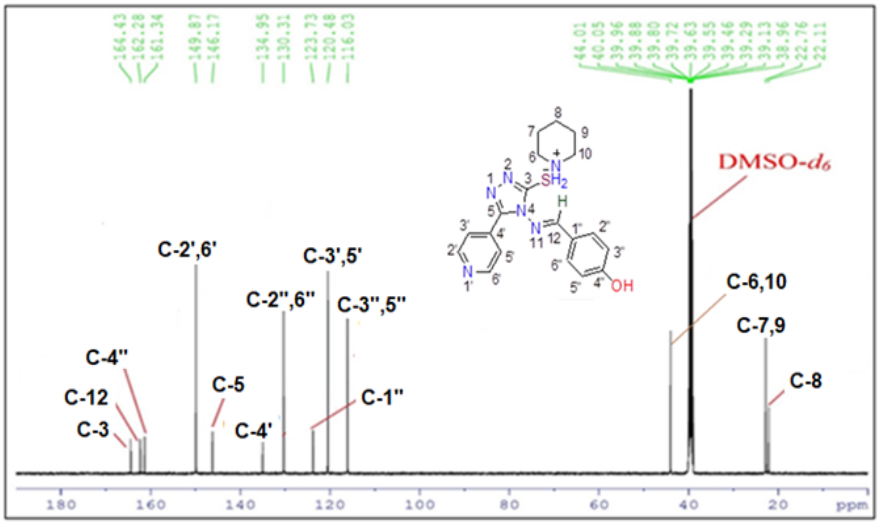


Figure 3: ^{13}C-NMR spectrum of **7Ai** (125 MHz, DMSO-d_6)

The methine and quaternary carbons in compound **7Ai** were confirmed using DEPT-135 NMR spectrum (Figure 4). The 2D-NMR correlation using ^{1}H-^{1}H COSY (Figure 5) and ^{1}H-^{13}C HMQC (Figure 6) spectra confirmed the assignment of the aromatic protons. The COSY spectra of all the Schiff base derivatives, **7A(i-viii)** showed clear correlations of the neighboring protons between H-2'/H-6' and H-3'/H-5' in Ring 1 (aryl group) and H-2"/H-6" and H-3"/H-5" ring 2 (phenyl group). Further confirmation of **7Ai** using ^{1}H-^{13}C HMBC (Figure 7) showed the short-range correlations between the carbon atoms of the aryl/phenyl. All the synthesized Schiff base compounds have two aromatic rings and one piperidinium ring. Two substituents attached to the benzene ring were the aryl group (pyridin-4-yl and 4-hydroxyphenyl) of the triazole ring (Ring 1) and a phenyl group of the aromatic aldehyde (Ring 2). In conclusion, the spectroscopic data led to the clear identification of all the compounds.

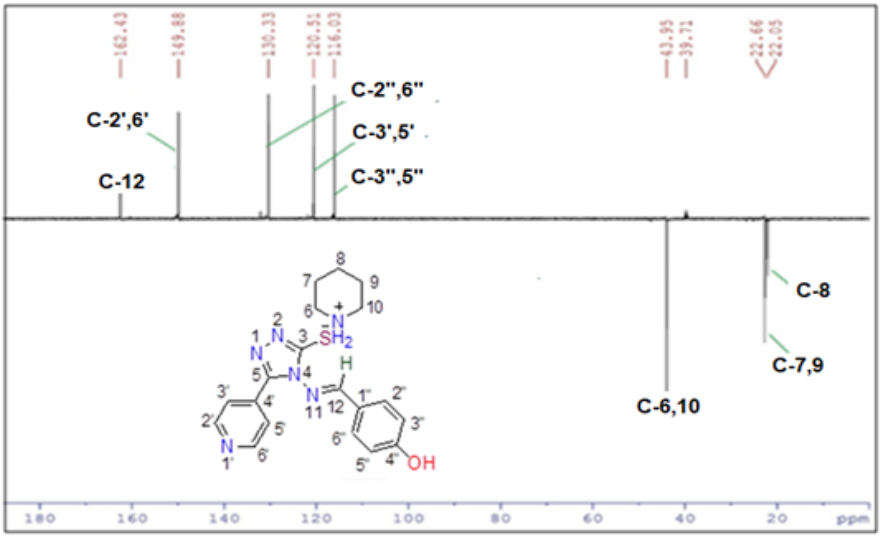


Figure 4: DEPT-135 NMR spectrum of **7Ai** (125 MHz, DMSO-d_6)

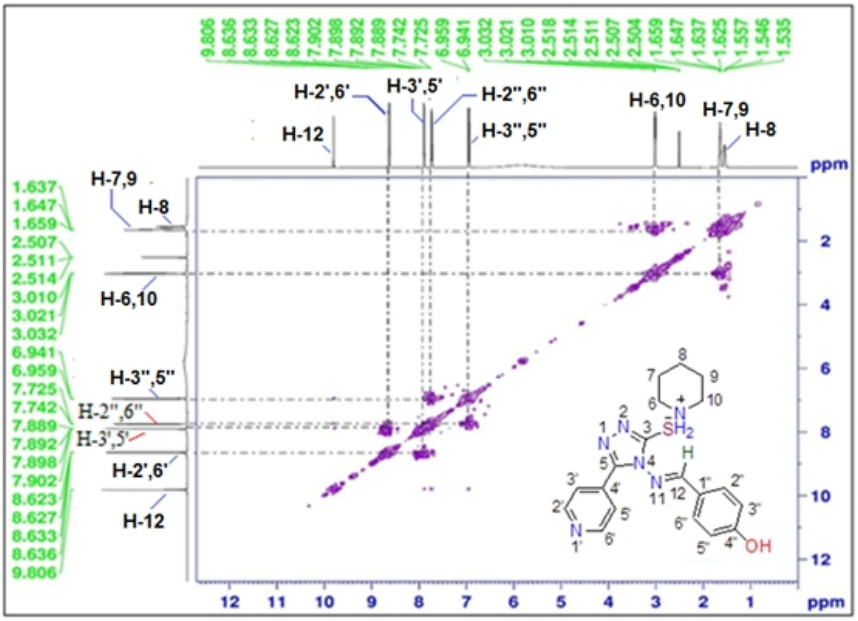


Figure 5: ^{1}H-^{1}H COSY NMR spectrum of **7Ai** (500 MHz, DMSO-d_6)

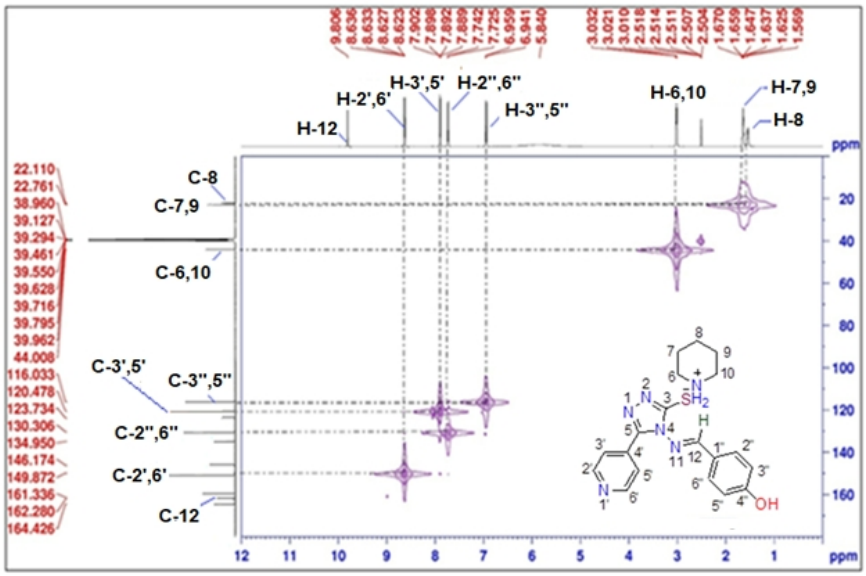


Figure 6: ^{1}H-^{13}C HMQC NMR spectrum of **7Ai (**500 MHz, DMSO-d_6)

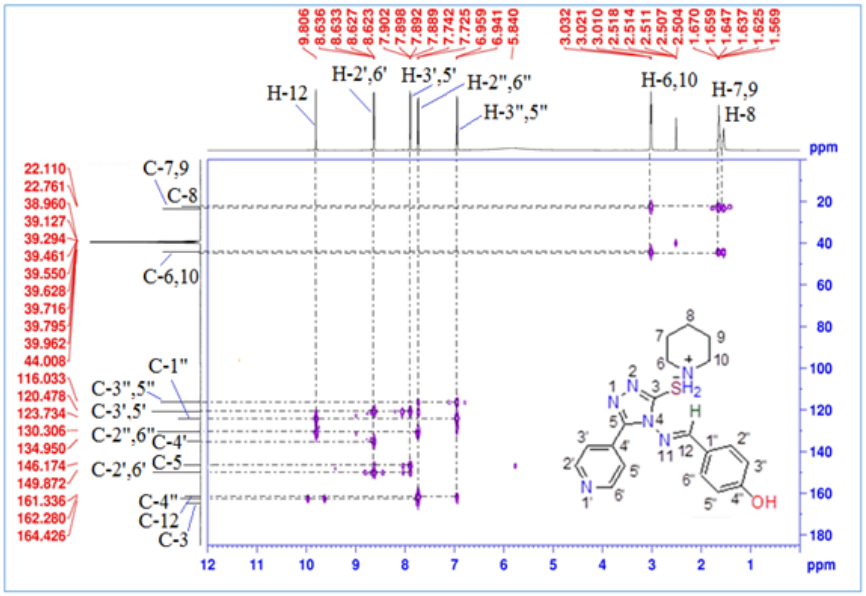


Figure 7: ^{1}H-^{13}C HMBC NMR spectrum of **7Ai (**500 MHz, DMSO-d_6)

2.2 Cytotoxicity study

Compounds of the mono system with different moieties attached to the triazole ring were tested for cytotoxicity activity against two different cell lines of human breast cancer (MCF-7) cell and human colorectal cancer (HCT-116) cell. The IC_{50} values of these

compounds were compared with the standard references used, Tamoxifen as a positive control for MCF-7 and 5-fluorouracil as a positive control for HCT-116. Cytotoxic activity is an ability of a test sample to cause toxic effects in the cancer cells which can be measured by assessing the proliferation of the cells [27-30]. The dose-dependent antiproliferative effect of the compounds tested against human breast tumor cells (MCF-7) is presented in Figure 8.

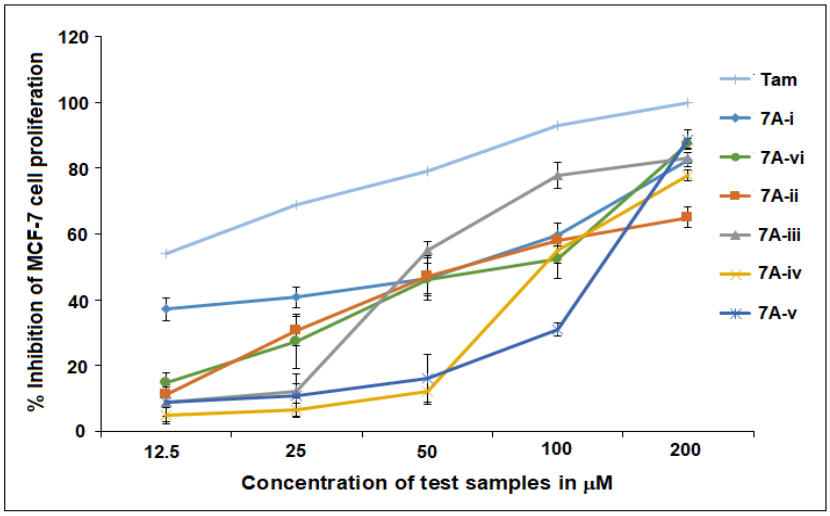


Figure 8: Graphical illustration of the dose-pendent response of the compounds tested on human breast cancer cells (MCF-7). The data is presented as mean percentage inhibition ± SD (n = 3). Tam = tamoxifen.

Figure 9 shows the human breast cancer (MCF-7) cell images observed under an inverted phase-contrast microscope attached to a digital camera, after 48 hours being treated with compounds **7A(i-vi)**. Tamoxifen was used as the reference standard and treated cell group with 0.1% DMSO used as the negative control. Cells from the negative control group exhibited full confluent compact monolayer of the proliferating MCF-7 cells. However, cells treated with the standard drug, tamoxifen (IC_{50} = 8.2 µM) demonstrated significant ($p < 0.01$) inhibitory effect on the proliferation of the cells. Images of the standard-treated cells showed clear toxic signs such as reduced cellular population, round shaped cells and loss of the pseudopodial cellular projections. Pseudopodial projections are the cytoplasm-filled

projection of the cell membrane. These projections help the cells to get attached to another cell or a surface of the tissue or flask [31]. Basically, these cellular projections originate with the help of cytoskeleton of the cells to support and maintain the structural features of the cells [32]. Among the compounds tested, compound **7Ai** demonstrated significantly potent inhibitory effect (IC_{50} 38 μM) on cellular proliferation, as the cellular population was reduced drastically. Compounds **7Aii**, **7Av** and **7Avi** demonstrated the considerable cytotoxic activity with IC_{50} of 68, 85 and 53 μM, respectively. On the other hand, compounds **7Aiii** and **7Aiv** exhibited a moderate inhibitory effect on the proliferation of human breast cancer cells with IC_{50} 91 and 101 μM, respectively.

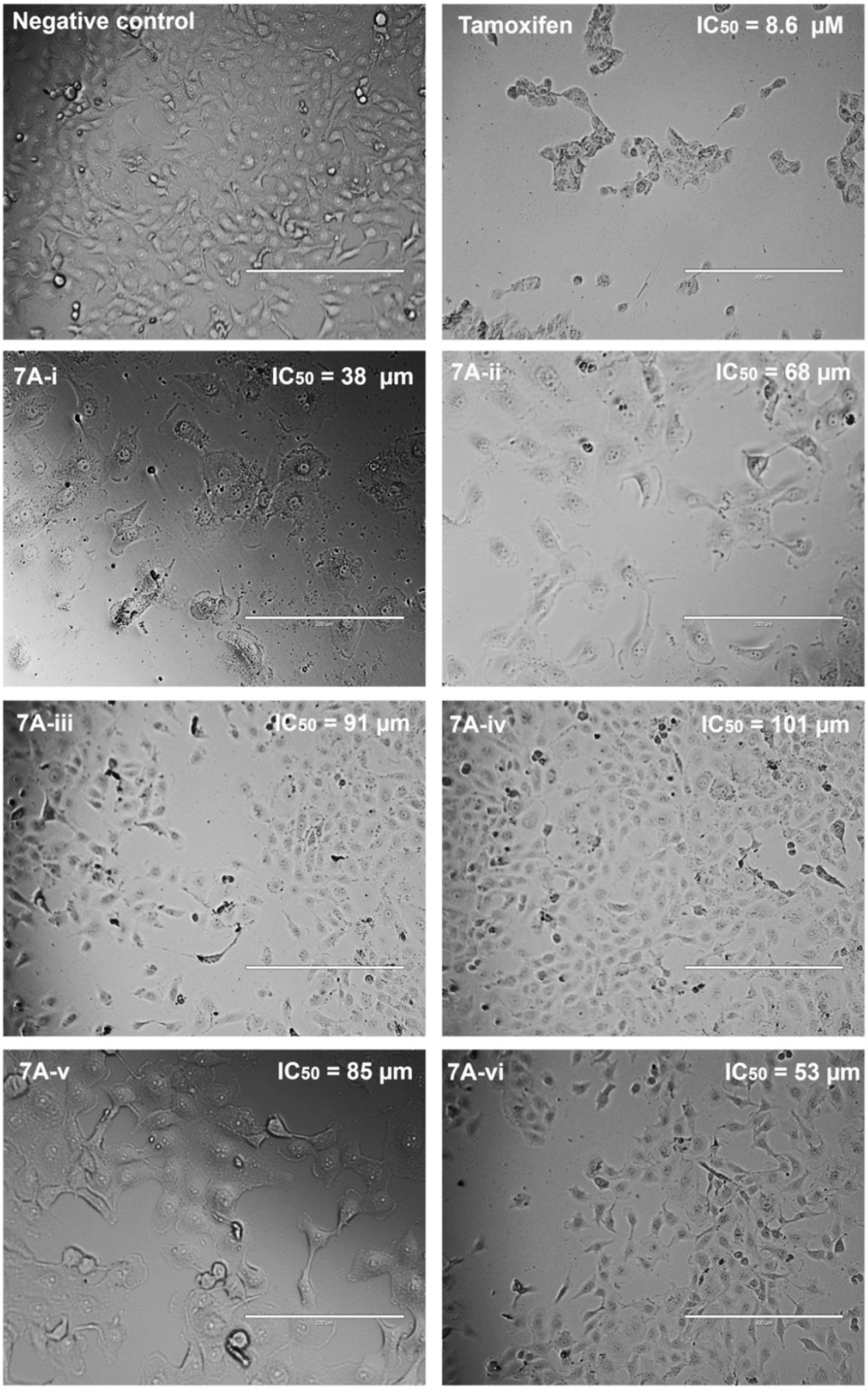


Figure 9: Images of human breast cancer (MCF-7) cell under an inverted phase-contrast microscope with a digital camera at 48 hours after treatment with compounds **7A(i-vi)**.

Tamoxifen is the reference standard and treated cell group with 0.1 DMSO as a negative control (Magnification × 200).

The dose-dependent antiproliferative effect of the compounds tested against human colorectal tumor cells (HCT-116) is graphically presented in Figure 10.

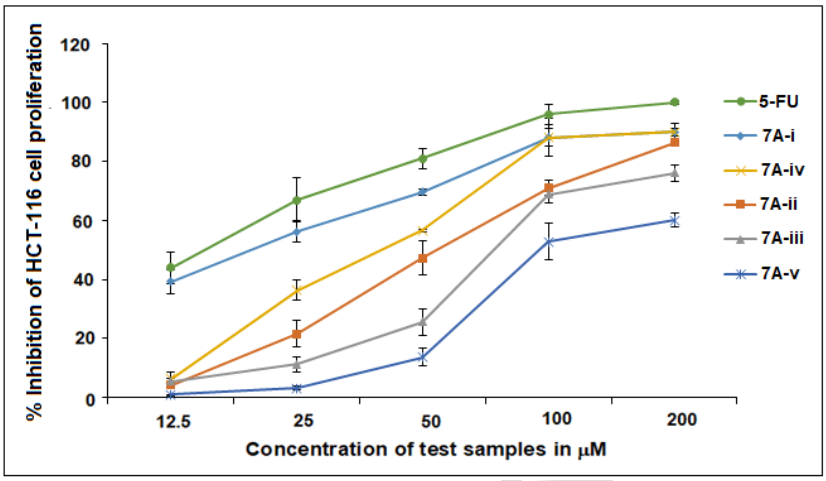


Figure 10: Graphical illustration of the dose-pendent response of the compounds tested on human colorectal tumor cells (HCT-116). The data is presented as mean percentage inhibition ± SD (n = 3). 5-FU = 5-fluorouracil.

Photomicrographic images (Figure 11) of human colorectal tumor (HCT-116) cell were captured using a digital camera observed under an inverted phase-contrast microscope at magnification power of × 200. The images were taken at 48 hours after the treatment. The cells from the negative control group (treated with 0.1% DMSO) displayed a complete confluent monolayer of aggressively growing HCT-116 cells while the cells from the positive control group (treated with the standard drug 5-fluorouracil) demonstrated significant inhibitory effect ($p<0.01$) on the proliferation of the cells with IC_{50} 6.7 µM. The standard reference, 5-fluorouracil caused the manifestation of obvious toxic signs in the treated cells, such as round shaped morphology and affected the normal pseudopodial like cellular projections in the cells. Among the synthetic compounds tested in this study, **7Ai** revealed

more pronounced antiproliferative effects with $IC_{50:}$ 19.2 μM. The cellular morphological analysis revealed that compound **7Ai** might have caused autophagy in the treated cells, as it is clearly evident from plenty of autophagic vacuoles in the cytoplasm of the treated cells. Compounds **7Aii** and **7Avi** showed moderate cytotoxicity with IC_{50}: 59.3 and 41.2 μM, respectively. Similar to compounds **7Ai**, **7Aii** and **7Aiii**, autophagic vacuoles were also observed in the treated cells. On the other hand, compounds **7Aiii**, **7Aiv** and **7Av** demonstrated a moderate inhibitory effect on the proliferation with IC_{50}: 81.7, > 300 and 137.1 μM, respectively, as the population of cells was reduced considerably, however cellular morphology remained more or less similar to that of the negative control.

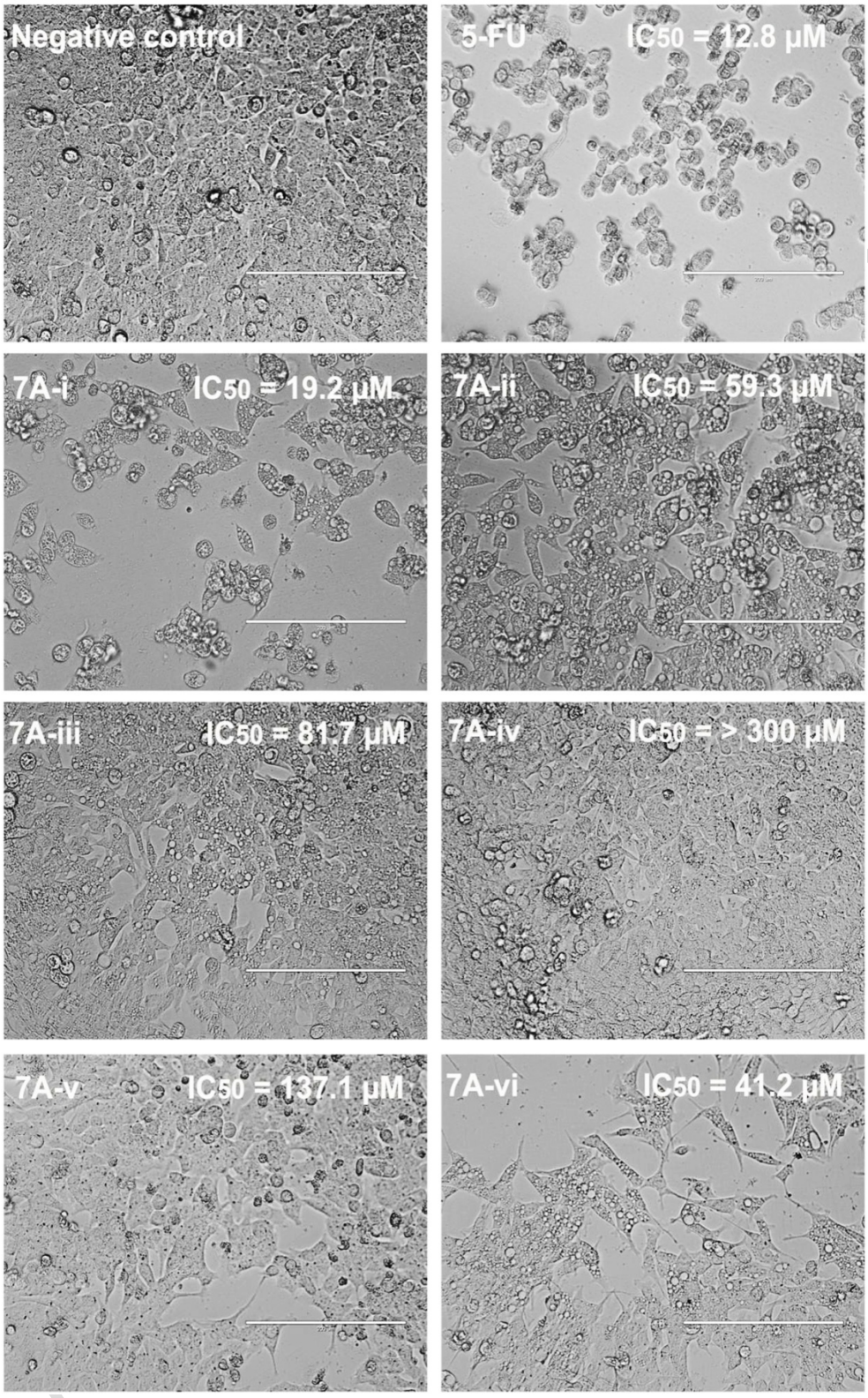


Figure 11: Photomicrographic images of human colorectal tumor (HCT-116) cells under an inverted phase-contrast microscope with a digital camera at 48 hours after treatment with

compounds **7A(i-vi)**. The reference standard is 5-fluorouracil (5-FU) and treated cell group with 0.1% DMSO as a negative control (Magnification × 200).

All the IC_{50} values of compounds **5(A-B), 6(A-B), 7A(i-viii)** and **7B(i-viii)** on MCF-7 and HCT-116 cancer cell lines are summarized in Table 1.

Table 1: IC_{50} values of the synthesized compounds against MCF-7 and HCT-116 cancer cell lines. Each value is the average of triplicate experiments with standard deviation

Compounds	**IC_{50} values (μM) on the cancer cells**	
	MCF-7	**HCT-116**
5A	92	74.1
5B	231	366.2
6A	87	112.4
6B	>300	184.6
7Ai	38	19.2
7Aii	68	59.3
7Aiii	91	81.7
7Aiv	101	>300
7Av	85	137.1
7Avi	53	41.2
7Avii	>300	>300
7Aviii	178.9	>300
7Bi	122.3	224
7Bii	106	132
7Biii	172	152.1
7Biv	>300	97.7
7Bv	>300	106.2
7Bvi	>300	155.6
7Bvii	>300	107.7
7Bviii	>300	211.43
Tamoxifen	8.8	
5-FU	--	12.8

2.3 Structure Activity Relationship (SAR)

The analysis of SARs correlates the effects of a chemical structure and its biological activity to develop the most effective drug. Through the analysis of the results obtained on human breast cancer cell MCF-7 and colon cancer cell HCT-116 (Table 1), it is possible to deduce some relationships between the structures of these newly synthesized compounds and their measured cytotoxicity in order to optimize and identify a potent anti-tumor drug.

For drug candidates, the molecular configuration of chemical structure plays a highly crucial role in the therapeutic efficacy, as a relatively minor change in the structure can lead to major changes in medicinal efficacy [33]. So, in this study an attempt is made to recognize the functional groups which are important for pronounce activity. At first stage, 2 derivatives of 1,2,4-triazole were synthesized with pyridine (**5A**) and phenolic (**5B**) rings. The results of the study revealed an interesting phenomenon with respect to presence of the adjacent pyridine or phenolic ring at the 5th carbon of 1,2,4-triazole nucleus. Pyridine ring with 1,2,4-triazole (**5A**) showed to have cytotoxic activity against both the cell lines (MCF-7 and HCT 116), whereas, phenolic ring with 1,2,4-triazole (**5B**) showed poor cytotoxic effect against the cell lines. Further structural modifications was done by attaching a piperidine moiety to the thiol group of 1,2,4-triazole of **5A** and **5B**. However, the biological activity of the compounds was not much affected, as the cytotoxicity of the compounds (**6A** and **6B**) remains same as that of their parental compounds (**5A** and **5B**). In the next stage, Schiff base complexes were derived from **6A** and **6B** by adding either benzaldehyde or 1-methyl-pyrrolidin-2-one to the amino group. Consistently, the results for the B-series compounds (with phenolic ring at 5th carbon of 1,2,4-triazole) showed no improvement in the cytotoxic effect, as all the compounds of the B-series remain poorly cytotoxic. Note worthily, addition of methyl pyrrolidin functional group to 1,2,4-triazole-pyridin complex, which yielded the Schiff base **7Aviii**, resulted in loss of cytotoxic activity of the compound. Addition of benzaldehyde to amino group of 1,2,4-

triazole – pyridine complex and attaching different functional groups at the 4th carbon of the benzaldehyde resulted in a series of compounds (**7Ai** to **7Avii**) with differential biological activity. Among the compounds, **7Ai** found to be the most potent cytotoxic compound.

Findings from the MTT assay against human breast tumor cells (MCF-7) showed that compounds **5A**, **6A** and **7A(ii-vi)** exhibited moderate anti-proliferation activity while compounds **5B, 6B**, and **7B(iv-viii)** showed much weaker activity. The other remaining compounds were considered inactive for MTT assay against MCF-7 cells, hence the IC_{50} values for such compounds could not be determined. The MTT assay against human colorectal tumor (HCT-116) cell showed that compounds **7Ai** and **7Avi** demonstrated significant cytotoxic effect with IC_{50} 19.2 and 41.2, respectively, while compounds **6A, 6B, 7Aiii, 7Av** and **7B(ii-viii)** showed moderate to weaker activity while other compounds showed poor activity with the IC_{50} of > 200, as shown in Table 1.

The results suggested that the hydroxyphenyl ring of piperidinium-4*H*-1,2,4-triazole-3-thiolate strongly affected the activity. Furthermore, changing the hydroxyphenyl ring with a pyridyl ring led to the loss of antiproliferative activity against HCT 116 cells. These modifications and structure-activity relationship studies revealed that the hydroxyphenyl ring of piperidinium-4*H*-1,2,4-triazole-3-thiolate played a critical role in their anti-proliferation activity[34].

Furthermore, the results suggested that the hydroxyphenyl group at position-4 of 1,2,4-triazole ring attached to 5-hydroxyphenyl and 3-piperidinium-4*H*-thiolate affected the activity. Changing the *p*-hydroxyphenyl ring (**7Ai**) with *p*-cyanide phenyl ring **(7Avi)** led to the loss of antiproliferative activity against MCF-7 cells. An overview of the structure-cytotoxicity activity relationship study can be highlighted as follows: (i) The 1,2,4-triazole ring framework is an important pharmacophore for the design and synthesis of novel anticancer agents with significant pharmacological activity and low toxicity. (ii) The

introduction of the substituted aromatic moiety at position-5, piperidinium-4*H*-thiolate at position -3 and a series of *para*-substituted phenyl ring at position-4 played a crucial role in modulating the anticancer activities of 1,2,4-triazole derivatives. (iii) Different substituents at position-4 (hydroxy and cyanide) are suitable candidates as powerful cytotoxic agents.

The most probable reason for the enhanced cytotoxicity of **7Ai** can be attributed to its polarity. Comparatively, the compound **7Ai** has a polar functional group (hydroxyl group), whereas the compounds of the series have non-poplar groups, such as methoxy (OCH_3), methyl (CH_3), chloro (Cl), bromo (Br), nitrile/cyano (CN) and nitro (NO_2) groups. Unlike the other compounds of the series, **7Ai** has both hydrophobic side groups (pyridine and piperidine) as well as a hydrophilic group (hydroxyl). Because of this, the compound **7Ai** may exhibit amphipathic property in biological system which enhanced the penetrability of the compound through the cell membranes and allows the compound to effectively interact with biomolecules such as proteins, RNA and DNA.

3. Experimental

3.1 Chemicals

The chemicals and reagents used in the syntheses, characterisation and application work of all the synthesised compounds include acetic acid glacial, acetone, chloroform, dichloromethane, diethyl ether, ethyl acetate, *n*-hexane, toluene, tetrahydrofuran,methanol, (99.5%), ethanol (99.7%) dimethyl sulfoxide-d_6, chloroform-*d,* sulphuric acid (95-97%), hydrochloric acid (37%), pyridine (99.5%), piperidine (99%), carbon disulphide (99%) hydrazine monohydrate (80%) 4-pyridinecarboxylic acid hydrazide (99%), 4-hydroxybenzhydrazide (98%), *p*-tolualdehyde (98%), anisaldehyde (98%), 3-hydroxybenzaldehyde (97%), 4-cyanobenzaldehyde (97%), *p*-nitrobenzaldehyde (99%), magnesium sulphate anhydrous, methanol-d_4, potassium bromide (FT-IR grade), sodium

hydrogen carbonate, calcium chloride anhydrous, potassium hydroxide, sodium hydroxide, methyl Paraben, TLC silica gel 60 F254 (aluminium sheet, 20 cm × 20 cm). These chemicals and solvent were purchased from Merck (Germany), BDH (England), Sigma-Aldrich (USA), Aldrich Chemicals Co Ltd (England), Fluka (Germany), System, UNILAB (Sydney, Australia), Fisher Scientific (UK), ARMAR Chemicals (Switzerland), R & M Chemicals (UK) and QRëC. All chemical were used directly without further purifications.

3.2 Instruments

All the instruments used for the characterization work are located at the School of Chemical Sciences, Universiti Sains Malaysia, Penang, Malaysia. Infrared Spectroscopy (IR): Samples were prepared as potassium bromide (KBr) discs and were recorded on a Perkin-Elmer System 2000 FT-IR spectrometer. All the 1D and 2D NMR spectra were recorded on a Bruker Avance 500 MHz. The elemental analyses were executed on Perkin Elmer II, 2400 CHN analyzer. The instrument for the cytotoxic assay used includes NuAire Biosafety cabinet class II, from NuAire (USA). Galaxy® CO_2 incubator from RS Biotech (UK). Axiovert 25 inverted phase-contrast microscope from Carl Zeiss (Germany). FM-2034 High-Quality Elisa Reader machine (USA). AMG EVOS fI inverted microscope from Electron Microscopy Sciences (USA).

3.3 Cytotoxicity assay

The reagents and chemicals used include DMEM and RPMI-1640 medium with L-glutamine, heat-inactivated foetal bovine serum (HIFBS), penicillin (10000 U/mL) – streptomycin (10 mg/mL) and trypsin-EDTA (10 ×) from GIBCO (USA). Phosphate buffered saline (PBS) tablets and sodium hydrogen carbonate from Sigma-Aldrich (USA). Dimethylsulfoxide from Fisher Scientific (UK), 3-[4,5-dimethylthiazol-2-yl]-2,5-

diphenyltetrazolium bromide (MTT) from PhytoTechnology Laboratories (USA). The tissue culture materials include tissue culture flasks (25 cm^2 and 75 cm^2) with 0.2 µm polysulphone filter cap, 96-well flat bottom tissue culture plates, 10 mL serological pipettes, vacuum filtration system with PES membrane, syringe filters (0.22 µm) and cryotubes from Techno Plastic Products (Switzerland) and 15 mL plastic centrifuge tubes from Corning Life Sciences (USA). The cell lines include estrogen-dependent human breast cancer (MCF-7) and human colorectal cancer (HCT-116) cell lines from the American Type Culture Collection (USA).

For cytotoxicity assay, stock solutions (10 mg/mL) of all the test samples were prepared in DMSO. Using serial dilutions of the stock solutions, various concentrations (12.5 to 200 µg/mL) of the test samples were prepared with cell culture media.

3.4 Synthesis method

3.4.1 General procedure for the synthesis of methyl 4-hydroxybenzoate, **1**

A mixture of 4-hydroxybenzoic acid (1.38 g, 0.01 mol) in 50 mL methanol and 5 mL concentrated sulphuric acid was refluxed and the reaction progress was monitored by TLC. After 12 hours, the mixture was poured into ice water and treated with 1N sodium bicarbonate solution to give a white precipitate. The product was filtered, dried and recrystallized from methanol.

Yield: (95%) as white crystalline; mp: 126-128°C (Lit: 125-128°C). Molecular weight: 152.15. IR (cm^{-1}): 3286 (O-H stretch); 3034 (C_{sp2}-H stretch); 2963 and 2855 (C_{sp3}-H stretch); 1677 (C=O); 1605 and 1512 (C=C aromatic); 1105 (C-O). ^{1}H-NMR (500 MHz, $CDCl_3$) δ, ppm: 7.98 (2H, d, *J*=8.60 Hz, H-2, H-6); 6.91 (2H, d, *J*=8.60 Hz, H-3, H-5); 3.93 (3H, s, H-8). ^{13}C NMR (125 MHz, $CDCl_3$) δ, ppm: 167.5 (C-7); 160.3 (C-4); 133.0 (C-2, C-6); 122.3 (C-1); 115.3 (C-3, C-5); 52.1 (C-8).

3.4.2 General procedure for the synthesis of 4-hydroxybenzohydrazide, **2**

A mixture of methyl 4-hydroxybenzoate, **1** (15.0 g, 0.10 mol) and hydrazine hydrate 80% (9.0 g, 0.18 mol) in 15 ml methanol was refluxed for 8 h. The reaction mixture was then poured into ice water. Hydrochloric acid (10%) was added to neutralize the mixture. The precipitate formed was vacuum filtered and dried overnight in the oven at 55°C. The product was recrystallized from 90% ethanol. Yield: 11.46 g (76.4%), light brown crystal; mp: 262-264°C (Lit.: 266°C). IR (cm^{-1}): 3318 (O-H stretch); 3280 and 3197 (-NH_2); 3009 (C_{sp2}-H aromatic); 1619 (C=O); 1537 and 1467 (C=C aromatic); 1326 (C-N); 1255 (C-O). ^{1}H-NMR (500 MHz, pyridine-d_5) δ, ppm: 10.67 (1H, s, -OH); 8.28 (2H, d, *J*=10.0 Hz, H-2, H-6); 7.24 (2H, d, *J*=10.0 Hz, H-3, H-5); 5.23 (2H, s, -NH_2). ^{13}C-NMR (125 MHz, pyridine-d_5) δ, ppm: 168.8 (C-7); 162.4 (C-4); 130.4 (C-2, C-6); 125.8 (C-1); 116.5 (C-3, C-5).

3.4.3 General procedure for the synthesis of potassium 2-(4-hydroxybenzoyl) hydrazinecarbo-dithioate, **3**

4-Hydroxybenzohydrazide, **2** (0.10 mol) and potassium hydroxide (0.26 mol) were dissolved in 100 mL ethanol. The mixture was cooled in an ice bath before the dropwise addition of carbon disulphide (0.23 mol). The mixture was stirred for a day. The precipitate formed was collected by vacuum filtration, washed with anhydrous diethyl ether (100 mL) and dried in vacuum oven. Yield: 80.8%, white powder; mp: 280-282°C. Molecular weight: 266.38. IR (cm^{-1}): 3380 (O-H stretch); 3148 (N-H); 3016 (C_{sp2}-H aromatic); 1646 (C=O); 1605 and 1491 (C=C aromatic); 1330 (NCS); 1077 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 7.68 (2H, d, *J*=8.70 Hz, H-2, H-6); 6.85 (2H, d, *J*=8.70 Hz, H-3, H-5). ^{13}C-NMR (125 MHz, DMSO -d_6) δ, ppm: 168.2 (C-7); 160.5 (C-4); 129.8 (C-2, C-6); 128.7 (C-10); 122.6 (C-1); 115.7 (C-3, C-5). CHN Elemental analysis, Calculated for $C_8H_7KN_2O_2S_2$: C, 36.07; H, 2.65; N, 10.52 Found: C, 33.38; H, 2.45; N, 9.49.

3.4.4 General procedure for the synthesis of 4-Amino-5-(4-hydroxyphenyl)-4*H*-[1,2,4] triazole-3-thiol, **5**

A suspension of potassium 2-(4-hydroxybenzoyl)hydrazinecarbodithioate, **3** (0.10 mol), hydrazine hydrate, 80% (15 mL, 0.30 mol) and water (5 mL) was refluxed at 110-120^{o}C for 8 hours. H_2S evolved after a clear solution resulted. The mixture was diluted with cold water (25 mL) and subsequent acidification with dilute HCl formed a precipitate which was then filtered, washed with cold water and dried in the oven at 55^{o}C. Yield: 78.7%, white powder; mp: 263-265°C. Molecular weight: 208.24. IR (cm^{-1}): 3305 (O-H stretch); 3270 and 3100 (-NH_2); 3030 (C_{sp2}-H aromatic); 2591 (S-H); 1604 (C=N); 1512 and 1477 (C=C aromatic); 1435 (C-N); 1222, 1066 and 1003 (C-N-C); 944, 825 and 693 (1,2,4-triazole ring). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 13.77 (1H, s, -SH); 10.04 (1H, s, OH); 7.87 (2H, d, *J*=8.60 Hz, H-2', H-6'); 6.88 (2H, d, *J*=8.60 Hz, H-3', H-5'); 5.75 (2H, s, -NH_2). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 166.3 (C-3); 159.3 (C-4'); 149.5 (C-5); 129.6 (C-2', C-6'); 116.4 (C-1'); 115.2 (C-3', C-5'). CHN Elemental analysis: Calculated for $C_8H_8N_4OS$: C, 46.14; H, 3.87; N, 26.90. Found: C, 44.87; H, 3.59; N, 25.68.

3.4.5 General procedure for synthesis of piperidinium 4-amino-5-(4-sub-aryl)-4*H*-1,2,4-triazole-3-thiolate, **6(A-B)**

A mixture of compound **5A** or **5B** (0.01 mol) and piperidine (0.01 mol) in absolute methanol (50 mL) was refluxed for 3-5 hours. The reaction progress was monitored by TLC. A precipitate formed was filtered and dried. Purification using column chromatography gave a pure product.

Compound **6A**. Yield: (82.7%), khaki powder; mp:178-180°C. IR (cm^{-1}): 3418 (-$^{+}NH_2$ at piperidine ring); 3271 and 3160 (-NH_2 at triazole ring); 3058 (C_{sp2}-H aromatic); 2938 and 2852 (C_{sp3}-H aliphatic); 2435 (-$^{+}$NH at pyridine ring); 1605 (C=N); 1571 and 1478 (C=C

aromatic); 1413(C-N); 1217, 1085, 1003 (C-N-C); 937, 823, 687 (1,2,4-triazole ring). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 8.64 (2H, d, *J*=6.15 Hz, H-2', H-6'); 8.03 (2H, d, *J*=6.15 Hz, H-3', H-5'); 5.71 (2H, s, -NH_2); 5.31 (2H, br. s, -$^{+}NH_{2pip}$); 2.99 (4H, t, *J*=5.53 Hz, H-6, H-10); 1.62-1.64 (4H, m, H-7, H-9); 1.54-1.56 (2H, m, H-8). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 168.4 (C-3); 149.8 (C-2', C-6'); 146.2 (C-5); 134.8 (C-4'); 120.2 (C-3', C-5'); 44.3 (C-6, C-10); 23.2 (C-7, C-9); 22.1 (C-8).

Compound **6B**. Yield: (88.7%), orange powder; mp: 162-164°C. IR (cm-1): 3418 (-+NH_2 at piperidine ring); 3304 and 3208 (-NH_2 at triazole ring); 3304 (-OH, superimposed with the N-H bands); 3030 (C_{sp2}-H aromatic); 2927 and 2853 (C_{sp3}-H aliphatic); 1607 (C=N stretch); 1530 and 1464 (C=C aromatic); 1394 (C–N); 1253, 1057, 1015 (C-N-C); 945, 815, 690 (1,2,4-triazole ring). ^{1}H-NMR (500 MHz, DMSO-d6) δ, ppm: 7.86 (2H, d, *J*=8.25 Hz, H-2', H-6'); 6.88 (2H, d, *J*=8.25 Hz, H-3', H-5'); 6.37 (2H, br. s, -+NH_2 at piperidine ring); 5.67 (2H, s, -NH_2); 2.84 (4H, t, *J*=4.68 Hz, H-6, H-10); 1.51-1.53 (6H, m, H-7, H-8, H-9). ^{13}C-NMR (125 MHz, DMSO-d6) δ, ppm: 166.2 (C-3); 159.2 (C-4'); 149.1 (C-5); 129.1 (C-2', C-6'); 117.2 (C-1'); 115.3 (C-3', C-5'); 45.2 (C-6, C-10); 24.5 (C-7, C-9); 23.4(C-8).

3.4.6 General procedure piperidinium *(E)*-4-(4-sub-benzylideneamino)-5-(pyridin-4-yl)-4*H*-1,2,4-triazole-3-thiolate, **7A(i-viii)**

Schiff base compounds of piperidinium (*E*)-4-(4-sub-benzylideneamino)-5-(pyridin-4-yl)-4*H*-1,2,4-triazole-3-thiolate, **7A(i-vii)** and piperidinium (*E*)-4-(1-methylpyrrolidin-2-ylideneamino)-5-(pyridin-4-yl)-4*H*-1,2,4-triazole-3-thiolate, **7Aviii** were prepared according to two new procedures. In the first procedure, piperidine was added in the system which undergoes dehydrohalogenation to produce cyclic imine. In the second procedure, 4-amino-5-(pyridin-4-yl)-4*H*-1,2,4-triazole-3-thiole, **5A** (0.0026 mol) in absolute methanol (50 mL) with

a few drops of piperidine (0.5 mL) were refluxed for 30 mins before the addition of various aromatic aldehydes (0.0026 mol) and the mixture was continued to reflux for 7 hours. A solid precipitate formed was filtered off, dried under vacuum overnight and purified to give (*E*)-4-(4-sub-benzylideneamino)-5-(pyridin-4yl)-4*H*-1,2,4-triazole-3-thiolate, **7A(i-vii)** and **7Aviii**. In Scheme 2, Schiff base compounds, **7A(i-viii)** can be synthesized from starting material **6A** using the first procedure or from starting material **5A** by using the second procedure.

Compound **7Ai**. Yield: 82.9%, khaki powder; mp:150-152°C. IR (cm^{-1}): 3331 (O-H stretch); 3001 (C_{sp2}-H aromatic); 2955 (C_{sp3}-H aliphatic); 1651 (C=N);1592 and 1514 (C=C aromatic); 710 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 9.80 (1H, s, H-12); 8.63 (2H, d, *J*=6.15 Hz, H-2', H-6'); 7.90 (2H, d, *J*=6.15 Hz, H-3', H-5'); 7.73 (2H, d, *J*=8.65 Hz, H-2", H-6"); 6.95 (2H, d, *J*=8.65 Hz, H-3", H-5"); 3.02 (4H, t, *J*=5.60 Hz, H-6, H-10); 1.63-1.65 (4H, m, H-7, H-9); 1.54-1.56 (2H, m, H-8). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 164.4 (C-3); 162.3 (C-12); 161.3 (C-4"); 149.9 (C-2', C-6'); 146.2 (C-5); 135.0 (C-4'); 130.3 (C-2", C-6"); 123.7 (C-1"); 120.5 (C-3', C-5'); 116.0 (C-3", C-5"); 44.0 (C-6, C-10); 22.8 (C-7, C-9); 22.1 (C-8). CHN Elemental analysis: Calculated for $C_{19}H_{22}N_6OS$: C, 59.66; H, 5.80; N, 21.97. Found: C, 58.76; H, 5.82; N, 21.55.

Compound **7Aii**. Yield: 92.6%, yellow-green powder; mp:182-184°C. IR (cm^{-1}): 3007 (C_{sp2}-H aromatic); 2934 (C_{sp3}-H aliphatic); 1599 (C=N);1568 and 1511 (C=C aromatic); 729 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 9.75 (1H, s, H-12); 8.69 (2H, d, *J*=6.15 Hz, H-2', H-6'); 7.88 (2H, d, *J*=6.15 Hz, H-3', H-5'); 7.87 (2H, d, *J*=8.82 Hz, H-2", H-6"); 7.12 (2H, d, *J*=8.82 Hz, H-3", H-5"); 3.86 (3H, s, -OCH_3); 3.05 (4H, t, *J*=5.65 Hz, H-6, H-10); 1.65-1.67 (4H, m, H-7, H-9); 1.54-1.56 (2H, m, H-8). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 164.6 (C-3); 163.6 (C-12); 162.7 (C-1"); 150.1 (C-2', C-6'); 146.3 (C-5); 133.8 (C-4'); 130.5 (C-

3", C-5"); 124.8 (C-4"); 121.1 (C-3', C-5'); 114.7 (C-2", C-6"); 55.5 (OCH_3); 43.7 (C-6, C-10); 22.3 (C-7, C-9); 21.7 (C-8).

Compound **7Aiii**. Yield: 91.4%, lemon powder; mp:155-157°C. IR (cm^{-1}): 3012 (C_{sp2}-H aromatic); 2947 (C_{sp3}-H aliphatic); 1599 (C=N); 1567 and 1511 (C=C aromatic); 718 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 10.05 (1H, s, H-12); 8.65 (2H, d, *J*=6.50 Hz, H-2', H-6'); 7.89 (2H, d, *J*=6.50 Hz, H-3', H-5'); 7.79 (2H, d, *J*=7.85 Hz, H-2", H-6"); 7.38 (2H, d, *J*=7.85 Hz, H-3", H-5"); 1.69 (3H, s, -CH_3); 3.05 (4H, t, *J*=5.48 Hz, H-6, H-10); 1.53-1.55 (4H, m, H-7, H-9); 1.06-1.08 (2H, m, H-8). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 164.1 (C-3); 162.1 (C-12); 149.9 (C-2', C-6'); 146.4 (C-5); 142.4 (C-1"); 134.6 (C-4'); 130.3 (C-4"); 129.7 (C-3", C-5"); 128.3 (C-2", C-6"); 120.8 (C-3', C-5'); 43.9 (C-6, C-10); 22.5 (C-7, C-9); 21.9 (C-8); 21.2 (-CH_3).

Compound **7Aiv**. Yield: 23.1%, lemon powder; mp: 208-211°C. IR (cm^{-1}): 3026 (C_{sp2}-H aromatic); 2936 (C_{sp3}-H aliphatic); 1603 (C=N); 1559 and 1475 (C=C aromatic); 725 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm:10.23 (1H, s, H-12); 8.65 (2H, d, *J*=6.20 Hz, H-2', H-6'); 7.87 (2H, d, *J*=6.30 Hz, H-3', H-5'); 7.90 (2H, d, *J*=8.50 Hz, H-2", H-6"); 7.63 (2H, d, *J*=8.50 Hz, H-3", H-5"); 3.04 (4H, t, *J*=5.70 Hz, H-6, H-10); 1.63-1.65 (4H, m, H-7, H-9); 1.54-1.56 (2H, m, H-8). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 163.6 (C-3); 162.0 (C-12); 150.0 (C-2', C-6'); 146.6 (C-5); 131.5 (C-4'); 129.3 (C-3", C-5"); 136.9 (C-4"); 130.1 (C-2", C-6"); 134.0 (C-1"); 121.2 (C-3', C-5'); 43.7 (C-6, C-10); 22.2 (C-7, C-9); 21.7 (C-8).CHN Elemental analysis: Calculated for $C_{19}H_{21}ClN_6S$: C, 56.92; H, 5.28; N, 20.96. Found: C, 57.14; H, 5.24; N, 20.99.

Compound **7Av**.Yield: 26.9%, banana powder; mp: 224-226°C. IR (cm^{-1}): 3025 (C_{sp2}-H aromatic); 2926 (C_{sp3}-H aliphatic); 1604 (C=N stretch); 1561 and 1477 (C=C aromatic); 724 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 10.16 (1H, s, H-12); 8.67 (2H, d, *J*=6.20 Hz, H-2', H-6'); 7.87 (2H, d, *J*=6.30 Hz, H-3', H-5'); 7.84 (2H, d, *J*=8.50 Hz, H-2", H-6"); 7.77 (2H, d, *J*=8.50 Hz, H-3", H-5"); 3.05 (4H, t, *J*=5.68 Hz, H-6, H-10); 1.65-1.67 (4H, m, H-7, H-9); 1.55-1.57 (2H, m, H-8). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 163.9 (C-3); 161.0 (C-12); 150.0 (C-2', C-6'); 146.5 (C-5); 134.2 (C-4'); 132.2 (C-3", C-5"); 132.1 (C-4"); 130.1 (C-2", C-6"); 125.6 (C-1"); 121.0 (C-3', C-5'); 43.7 (C-6, C-10); 22.3 (C-7, C-9); 21.7 (C-8).

Compound **7Avi**. **Yield**: 55.3%, peach powder; mp: 260°C. IR (cm^{-1}): 3033 (C_{sp2}-H aromatic); 2947 (C_{sp3}-H aliphatic); 2232 (C≡N stretch); 1601 (C=N stretch); 1547 and 1468 (C=C aromatic);713 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 10.70 (1H, s, H-12); 8.62 (2H, d, *J*=6.15 Hz, H-2', H-6'); 7.87 (2H, d, *J*=6.05 Hz, H-3', H-5'); 8.04 (2H, d, *J*=8.30 Hz, H-2", H-6"); 7.98 (2H, d, *J*=8.30 Hz, H-3", H-5"); 3.00 (4H, t, *J*=5.58 Hz, H-6, H-10); 1.58-1.60 (4H, m, H-7, H-9); 1.50-1.52 (2H, m, H-8). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 164.5 (C-3); 156.1 (C-12); 149.6 (C-2', C-6'); 14.7 (C-5); 135.3 (C-4'); 132.9 (C-3", C-5"); 113.1 (C-4"); 128.5 (C-2", C-6"); 138.2 (C-1"); 120.5 (C-3', C-5'); 119.4 (C-N); 47.4 (C-6, C-10); 25.2 (C-7, C-9); 19.2 (C-8).

Compound **7Avii**. Yield: 31.9%, dark-orange powder; mp: 242-244°C. IR (cm^{-1}): 3036 (C_{sp2}-H aromatic); 2928 (C_{sp3}-H aliphatic); 1606 (C=N stretch); 1581 and 1519 (C=C aromatic); 1429 and 1340 (-NO_2 asymmetrical and symmetrical stretching); 749 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 10.20 (1H, s, H-12); 8.40 (2H, d, *J*=8.50 Hz, H-3", H-5"); 8.20 (2H, d, *J*=8.50 Hz, H-2", H-6"); 7.88 (2H, d, *J*=5.50 Hz, H-3', H-5'); 8.76 (2H, d, *J*=5.50 Hz,

H-2', H-6'); 3.03 (4H, t, *J*=5.50 Hz, H-6, H-10); 1.63-1.65 (4H, m, H-7, H-9); 1.44-1.46 (2H, m, H-8). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 163.1 (C-3); 162.4 (C-12); 150.2 (C-2', C-6'); 149.5 (C-4"); 146.8 (C-5); 138.0 (C-1"); 132.9 (C-4'); 129.8 (C-2", C-6"); 124.3 (C-3", C-5"); 121.8 (C-3', C-5'); 47.3 (C-6, C-10); 22.2 (C-7, C-9); 21.6 (C-8).

Compound **7Aviii**.Yield: 27.2%, khaki solid; mp: 253-255°C. IR (cm^{-1}): 3021 (C_{sp2}-H aromatic); 2926 (C_{sp3}-H aliphatic); 1605 (C=N stretch); 1570 and 1511 (C=C aromatic); 736 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 8.76 (2H, d, *J*=6.10 Hz, H-2', H-6'); 8.29 (2H, d, *J*=6.15 Hz,H-3', H-5'); 3.29 (2H, t, *J*=7.05 Hz, H-13); 3.02 (t, *J*=5.63 Hz, H-6, H-10); 2.69 (3H, s, -CH_3); 2.18 (2H, t, *J*=8.08 Hz, H-15); 1.90-1.92 (2H, m, H-14); 1.62-1.64 (2H, m, H-8); 1.53-1.55 (4H, m, -H-7, H-9). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 176.6 (C-12); 167.7 (C-3); 150.1 (C-2', C-6'); 147.4 (C-5); 132.9 (C-4'); 121.6 (C-3', C-5'); 48.5 (C-13); 43.8 (C-6, C-10); 30.1 (C-15); 29.3 (-CH_3); 22.2 (C-7, C-9); 21.6 (C-8); 17.2 (C-14).

Compound **7Bi**. Yield: 81.5%, peach powder; mp:160-162°C. IR (cm^{-1}): 3173 (O-H stretch); 3022 (C-H aromatic); 2992 (C-H aliphatic); 1608 (C=N);1575 and 1515 (C=C aromatic); 725 (C-S) ; ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 9.34 (1H, s, H-12); 7.76 (2H, d, *J*=8.50 Hz, H-3", H-5"); 7.71 (2H, d, *J*=8.75 Hz, H-2', H-6'); 6.94 (2H, d, *J*=8.50 Hz, H-2", H-6"); 6.88 (2H, d, *J*=8.75 Hz, H-3', H-5'); 2.80 (t, *J*=4.95 Hz, H-6, H-10); 1.51-1.53 (H6, m, H-7, H-8, H-9). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 167.3 (C-3); 162.0 (C-12); 161.9 (C-4"); 159.5 (C-1'); 148.5 (C-5); 131.0 (C-2", C-6"); 129.8 (C-2', C-6'); 122.8 (C-1"); 116.2 (C-4'); 116.1 (C-3', C-5'); 115.4 (C-3", C-5"); 43.7 (C-6, C-10); 22.2 (C-7, C-9); 21.6 (C-8).

Compound **7Bii**. Yield: 65.8% yellow powder; mp: 154-156°C. IR (cm^{-1}): 3256 (O-H stretch), 3109 (C_{sp2}-H aromatic); 2932 (C_{sp3}-H aliphatic); 1607 (C=N stretch); 1570 and 1514

(C=C aromatic); 729 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 9.73 (1H, s, H-12); 7.82 (2H, d, *J*=8.50 Hz, H-2", H-6"); 7.70 (2H, d, *J*=9.00 Hz, H-2', H-6'); 7.10 (2H, d, *J*=8.50 Hz, H-3", H-5"); 6.85 (2H, d, *J*=9.00 Hz, H-3', H-5'); 3.86 (3H, s, -OCH_3); 2.83 (4H, t, *J*=5.16 Hz, H-6, H-10); 1.50-1.52 (6H, m, H-7, H-8, H-9). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 163.4 (C-12); 162.4 (C-3); 162.0 (C-4"); 158.7 (C-1'); 148.6 (C-5); 130.2 (C-2", C-6"); 129.2 (C-2', C-6'); 125.3 (C-1"); 117.8 (C-4'); 115.2 (C-3', C-5'); 114.6 (C-3", C-5"); 55.5 (OCH_3); 45.3 (C-6, C-10); 24.6 (C-7, C-9); 23.4 (C-8).CHN Elemental analysis: Calculated for $C_{21}H_{25}N_5O_2S$: C, 61.29; H, 6.12; N, 17.02. Found: C, 60.84; H, 5.64; N, 16.45.

Compound **7Biii**. Yield: 84.8%, milk powder; mp: 204-206°C. IR (cm^{-1}): 3000 (C_{sp2}-H aromatic); 2944 (C_{sp3}-H aliphatic); 1610 (C=N stretch); 1568 and1509 (C=C aromatic); 724 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 9.92 (1H, s, H-12); 7.75 (2H, d, *J*=8.50 Hz, H-2", H-6"); 7.36 (2H, d, *J*=8.50 Hz, H-3", H-5"); 7.70 (2H, d, *J*=9.00 Hz, H-2', H-6'); 6.86 (2H, d, *J*=9.00 Hz, H-3', H-5'); 2.39 (3H, s, -CH_3); 3.18 (4H, t, *J*=5.33 Hz, H-6, H-10); 1.59-1.61 (4H, m, H-7, H-9); 1.52-1.54 (2H, m, H-8). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 162.6 (C-12); 162.1 (C-3); 158.7 (C-1'); 148.9 (C-5); 142.1 (C-1"); 130.3 (C-4"); 129.6 (C-3", C-5"); 129.2 (C-2', C-6'); 128.2 (C-2", C-6"); 118.0 (C-4'); 115.3 (C-3', C-5'); 44.7 (C-6, C-10); 23.8 (C-7, C-9); 22.9 (C-8); 21.2 (-CH_3). CHN Elemental analysis: Calculated for $C_{21}H_{25}N_5OS$: C, 63.77; H, 6.37; N, 17.71. Found: C, 63.73; H, 6.02; N, 17.13.

Compound **7Biv**. Yield: 52.8%, banana powder; mp: 208-210°C. IR (cm^{-1}): 3001 (C_{sp2}-H aromatic); 2944 (C_{sp3}-H aliphatic); 1611 (C=N stretch); 1591 and 1490 (C=C aromatic); 712 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 10.12 (1H, s, H-12); 7.87 (2H, d, *J*=8.55 Hz, H-2", H-6"); 7.61 (2H, d, *J*=8.55 Hz, H-3", H-5"); 7.68 (2H, d, *J*=8.80 Hz, H-2', H-6'); 6.85 (2H, d, *J*=8.80 Hz, H-3', H-5'); 2.89 (4H, t, *J*=5.20 Hz, H-6, H-10); 1.55-1.57 (6H, m, H-7,

H-8, H-9). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 162.0 (C-3); 160.4 (C-12); 158.5 (C-1'); 149.0 (C-5); 136.4 (C-1"); 132.0 (C-4"); 129.8 (C-2", C-6"); 129.2 (C-2', C-6'); 129.2 (C-3", C-5"); 118.1 (C-4'); 115.2 (C-3', C-5'); 44.9 (C-6, C-10); 24.0 (C-7, C-9); 23.0 (C-8). CHN Elemental analysis: Calculated for $C_{20}H_{22}ClN_5OS$: C, 57.75; H, 5.33; N, 16.84. Found: C, 57.91; H, 5.05; N, 16.60.

Compound **7Bv**. Yield: 44.2%, lemon powder; mp: 228-230°C. IR (cm^{-1}): 3003 (C_{sp2}-H aromatic); 2941 (C_{sp3}-H aliphatic); 1611 (C=N stretch); 1589 and 1489 (C=C aromatic); 709 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 10.10 (1H, s, H-12); 7.80 (2H, d, *J*=8.65 Hz, H-2", H-6"); 7.75 (2H, d, *J*=8.65 Hz, H-3", H-5"); 7.68 (2H, d, *J*=8.80 Hz, H-2', H-6'); 6.85 (2H, d, *J*=8.80 Hz, H-3', H-5'); 2.88 (4H, t, *J*=5.02 Hz, H-6, H-10); 1.54-1.56 (6H, m, H-7, H-8, H-9). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 162.0 (C-3); 160.7 (C-12); 158.5 (C-1'); 149.0 (C-5); 132,3 (C-4"); 132.2 (C-3", C-5"); 130.0 (C-2", C-6"); 129.3 (C-2', C-6'); 125.4 (C-1"); 118.0 (C-4'); 115.2 (C-3', C-5'); 45.0 (C-6, C-10); 24.1 (C-7, C-9); 23.0 (C-8). CHN Elemental analysis: Calculated for $C_{20}H_{22}BrN_5OS$: C, 52.18; H, 4.82; N, 15.21. Found: C, 52.36; H, 4.57; N, 14.87.

Compound **7Bvi**. Yield: 21.6%, yellow powder; mp: 222-224°C. IR (cm^{-1}): 3256 (O-H stretch); 3122 (C_{sp2}-H aromatic); 2953 (C_{sp3}-H aliphatic); 2227 (C≡N stretch), 1611(C=N stretch); 1594 and 1508 (C=C aromatic); 727 (C-S); ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 10.0 (1H, s, H-12); 8.09 (2H, d, *J*=8.00 Hz, H-2", H-6"); 8.03 (2H, d, *J*=8.00 Hz, H-3", H-5"); 7.70 (2H, d, *J*=8.65 Hz, H-2', H-6'); 6.90 (2H, d, *J*=8.65 Hz, H-3', H-5'); 3.02 (4H, t, *J*=5.08 Hz, H-6, H-10); 1.60-1.62 (6H, m, H-7, H-8, H-9). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 163.4 (C-12); 161.9 (C-3); 159.6 (C-1'); 149.0 (C-5); 136.4 (C-1"); 133.1 (C-3", C-

5"); 130.0 (C-2", C-6"); 129.1 (C-2', C-6'); 118.3 (C-4"); 116.0 (C-4'); 115.5 (C-3', C-5'); 114.4 (-CN); 43.8 (C-6, C-10); 22.3 (C-7, C-9); 21.7 (C-8).

Compound **7Bvii**. Yield: 70.5%, maroon powder; mp: 224-226°C. IR (cm^{-1}): 3282 (C_{sp2}-H aromatic); 2945 (C_{sp3}-H aliphatic); 1610 (C=N stretch); 1591 and 1508 (C=C aromatic); 1437 and 1343 (NO_2 asymmetrical and symmetrical stretching); 725 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 10.09 (1H, s, H-12); 8.39 (2H, d, *J*=8.70 Hz, H-2", H-6"); 8.15 (2H, d, *J*=8.70 Hz, H-3", H-5"); 7.70 (2H, d, *J*=8.80 Hz, H-2', H-6'); 6.90 (2H, d, *J*=8.80 Hz, H-3', H-5'); 3.02 (4H, t, *J*=5.01 Hz, H-6, H-10); 1.62-1.64 (6H, m, H-7, H-8, H-9). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 162.7 (C-12); 161.9 (C-3); 159.6 (C-1'); 149.5 (C-4"); 149.1 (C-5); 138.0 (C-1"); 130.0 (C-3", C-5"); 129.7 (C-2', C-6'); 124.3 (C-2", C-6"); 116.0 (C-4'); 115.5 (C-3', C-5'); 40.1 (C-6, C-10); 22.2 (C-7, C-9); 21.7 (C-8).

Compound **7Bviii**. Yield: 61.8%, weat powder; mp: 252-254°C. IR (cm^{-1}): 3273 (O-H stretch); 3106 (C_{sp2}-H aromatic); 2971 (C_{sp3}-H aliphatic); 1609 (C=N stretch); 1596 and 1512 (C=C aromatic); 732 (C-S). ^{1}H-NMR (500 MHz, DMSO-d_6) δ, ppm: 7.86 (2H, d, J=8.70 Hz, H-2', H-6'); 6.88 (2H, d, J=8.70 Hz,H-3', H-5'); 6.37 (br. s, -$^+NH_2$ at piperidine); 3.30 (2H, t, *J*=7.05 Hz, H-13); 2.82 (4H, t, *J*=4.50Hz, H-6, H-10); 2.70 (3H, s, -CH_3); 2.18 (2H, t, *J*=8.10 Hz, H-15); 1.90-1.92 (2H, m, H-14); 1.50-1.52 (6H, m, H-7, H-8, H-9). ^{13}C-NMR (125 MHz, DMSO-d_6) δ, ppm: 173.8 (C-12); 166.2 (C-3); 159.3 (C-1'); 149.1 (C-5);129.1 (C-2', C-6');117.2 (C-4'); 115.2 (C-3', C-5'); 48.5 (C-13); 45.3 (C-6, C-10); 30.1 (C-15); 29.0 (-CH_{3p}); 24.6 (C-7, C-9); 23.5 (C-8); 17.2 (C-14).

4. Conclusion

In this study, 18 new 1,2,4-triazole compounds with mono core system attached to different substituents have been synthesized by various methodologies. The first mono system showed two compounds of a piperidinium salt moiety attached to the triazole ring, **6(A-B)** while the second mono system showed 16 compounds of a piperidinium salt and a Schiff base moiety attached to the triazole ring, **7A(i-viii)** and **7B(i-viii)**. The structures of all these compounds were successfully characterized. The MTT assay against human breast cancer cells (MCF-7) showed that compounds **5A**, **6A** and **7A(ii-vi)** exhibited moderate anti-proliferation activity while compounds **5B, 6B**, and **7B(iv-viii)** showed much weaker activity. The other remaining compounds were inactive for MTT assay against MCF-7 cells, hence the IC_{50} values for such compounds could not be determined. The MTT assay against human colorectal cancer (HCT-116) cell showed that compounds **7Ai** and **7Avi** demonstrated moderate cytotoxic effect with IC_{50} 19.2 and 41.2 μM, respectively, while compounds **7Aii** and **5A** demonstrated the lesser inhibitory effect on cell proliferation. Compounds **6A, 6B, 7Aiii, 7Av** and **7B(ii-viii)** showed moderate to weaker activity while other compounds showed poor activity with the IC_{50} of > 200. The mono system of compounds **7Ai and 7Avi** showed potential cytotoxicity against human colorectal (HCT-116) and breast (MCF-7) cancer cells compared to other compounds of the mono system. Further modification on this scaffold based on the structure-property relationship helps in the design of better and effective compounds with improved anticancer activity.

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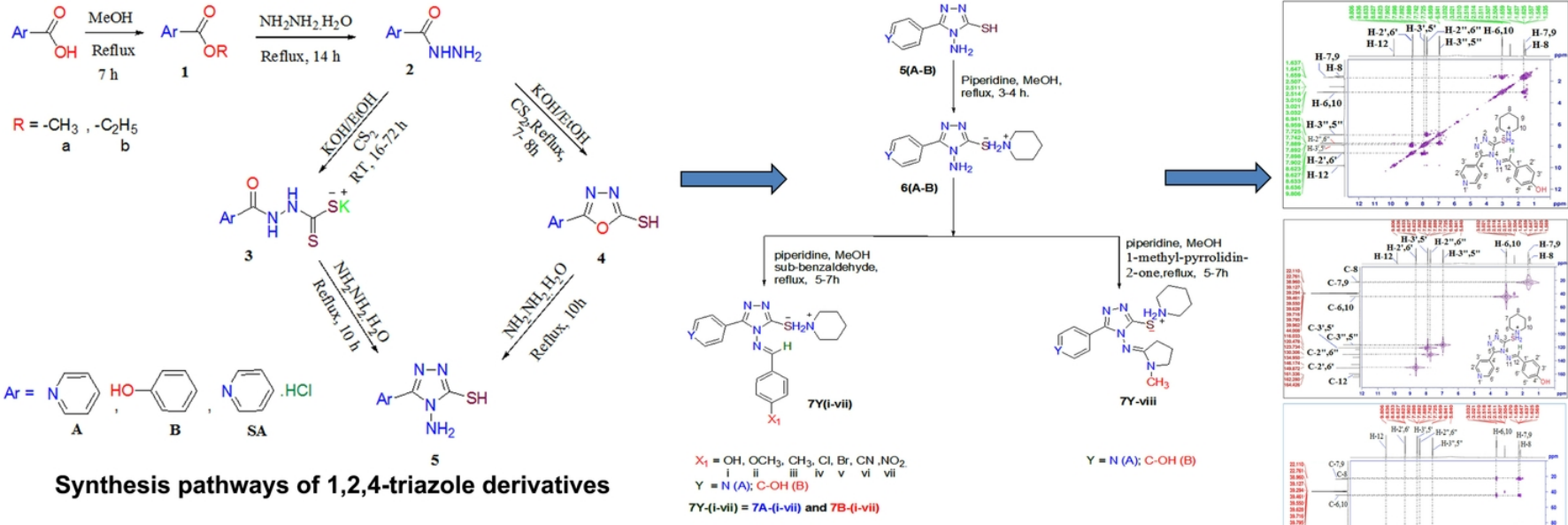
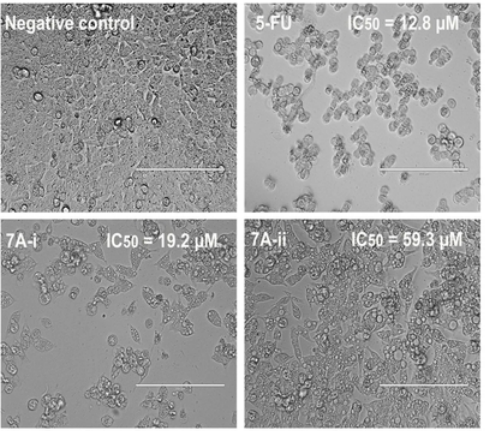
Highlights:

- 18 new 1,2,4-triazole compounds were synthesized and fully characterized.
- Compounds 7Ai and 7Avi demonstrated cytotoxic effect (IC_{50} 19.2 and 41.2 μM) against human colorectal cancer (HCT-116) cell, respectively.
- Compound 7Ai showed significantly antiproliferative effect (IC_{50} 38 μM) against MCF-7.



Synthesis pathways of 1,2,4-triazole derivatives

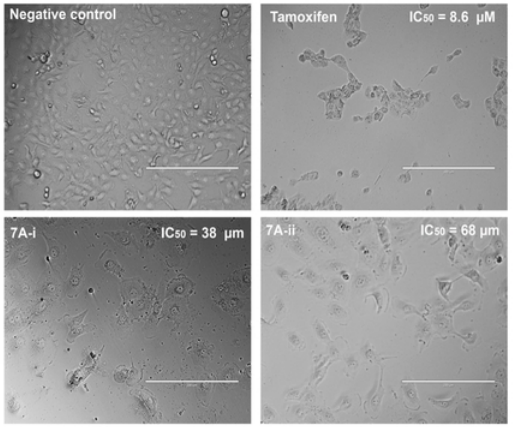
NMR studies



Anti-colon cancer study

Compounds	IC_{50} values (µM) on the cancer cells	
	MCF-7	HCT 116
5A	92	74.1
7A-i	38	19.2
7A-ii	68	59.3
7A-iii	91	81.7
7A-iv	101	>300
7A-v	85	137.1
7A-vi	53	41.2
Tamoxifen	8.8	
5-FU	--	12.8

IC_{50} values



Anti-breast cancer study