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Synthesis and In vetro Antiproliferative Evaluation of New Synthesized 1,3,4-Thiadiazole-Based Heterocycles

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Abstract:
Background*: 1,3,4-Thiadiazoles display a wide spectrum of biological activities including anticancer, antimicrobial, antiviral, antiepileptic, antidiabetic, analgesic, and anti-inflammatory activities. In particular, recent studies have pointed out the significance of the 1,3,4-thiadiazole scaffold in the field of current cancer research.*
Materials and Methods*: Novel 2,5-bis thiacarbohydrazono-1,3,4-thiadiazoles were designed, synthesized and their chemical structures were elucidated on the basis of elemental analyses and spectral data (FT-IR, ^{1}HNMR, ^{13}CNMR, mass spectra). In vitro antiproliferative activities were evaluated by viability assay against three types of human cancer cell lines, HepG-2, HCT-116 and MCF-7.*
Results*: 2,5-bis(mercapto-acetic-hydrazide)-1,3,4-thiadiazole was obtained from the precursor 2,5-dimercapto-1,3,4-thiadiazole, which in turn react with different aldehydes to afford the corresponding bis thiacarbohydrazono- 1,3,4-thiadiazoles in high yield. The relationships between the structures of synthesized compounds and their antiproliferative activity were investigated from their IC_{50} values.*
Conclusion: *New 2,5-bis thiacarbohydrazono-1,3,4-thiadiazoles were designed, synthesized and evaluated for antiproliferative activity against cancer cell lines; HepG-2, HCT-116 and MCF-7 in terms of IC_{50}.*
Key Word*: Thiadiazole; Bis carbohydrazides; Bis carbohydrazones; Antiproliferative; Viability; HepG-2; HCT-116 and MCF-7 cell lines.*

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I. Introduction

1,3,4-Oxadiazoles and thiazoles are versatile leading molecules for designing potential bioactive agents[1]. Compounds containing 1,3,4-oxadiazole cores have a broad biological activity spectrum including antimicrobial[1-8], anti-cancer[9-14], anti-inflammatory[15-17], antitubercular[18,19], anticonvulsant[20], antihypoglycemic[21], antimalarial[22,23] antiviral[24], vasodialatory[25,26], hypo-lipidemic[25,27] and ulcerogenic[25,28] activities as well as potential antihypertensive agents[29], and insecticidal[30] properties.

On the other hand, 1,3,4-thiadiazole derivatives exhibited a wide range of biological activities including antimicrobial, antituberculosis, anticonvulsant, anti-inflammatory, anti-cancer, and antiulcer, antileishmanial, analgesic, CNS depressant, antioxidant, antidiabetic, molluscicidal, antihypertensive, diuretic, analgesic, antitubercular properties[31-47]. Merging these two bioactive components; 1,3,4-oxadiazole and 1,3,4-thiadiazole, in one system might enhance the biological activity of the products. As part of our research work toward synthesis of heterocycles with broad biological activities[48-51], this work aims to synthesis new 1,3,4-thiadiazole-based heterocycles in order to study their antiproliferative activity against the HepG-2 (hepatocellular carcinoma), HCT-116 (colon carcinoma) and MCF-7 cells (human breast cancer) cell lines.

II. Material And Methods

Chemistry part, Melting points were determined with a Melt-temperature apparatus and are uncorrected. TLC was performed on Baker-Flex silica gel 1B-F plates and the spots were detected by UV light absorption. IR spectra were recorded on Perkin Elmer. USA Spectrometer. ^{1}H NMR, ^{13}C NMR were recorded on JEOL ECA-500 II (faculty of Science, AL Mansoura University, AL Mansoura, Egypt) using tetramethylsilane as an internal standard. Mass spectra were recorded on GCMS solution DI Analysis Shimadzu Qp-2010 Plus, at the faculty of Science, Cairo University, Cairo, Egypt. The ChemDraw-Ultra-8.0 has been used in generating the nomenclature of the prepared compounds.

Procedure methodology (Chemistry part)

Synthesis of *biscarbohydrazones* **4-6**. General Methods. A mixture of 2,5-bis(mercapto-acetohydrazide)-1,3,4-thiadiazole **3**[52] (2 mmoL) and aldehyde derivative (2 mmoL) is heated under reflux in ethanol (10 mL) and drops of acetic acid for 3 hour, the derivatives **4-6** that separated were filtered off and dried.

Bis ethyl 5-((2-(1,3,4-thiadiazol-2,5-diylthio)acetoylimino)methyl)-2-methylfuran-3-carboxylate **4**. It was obtained from 2,5-bis(mercapto-acetohydrazide)-1,3,4-thiadiazole **3** (2 mmoL) and 5-methyl-4-propionylfuran-2-carbaldehyde (2 mmoL). Recrystallization from DMSO as white crystals.

Bis 2-(1,3,4-thiadiazol-2,5-diylthio)-N'-((2-phenyl-2H-1,2,3-triazol-4-yl)methylene)acetohydrazide **5.** It was obtained from 2,5-bis(mercapto-acetohydrazide)-1,3,4-thiadiazole **3** (2 mmoL) and 2-phenyl-2*H*-1,2,3-triazole-4-carbaldehyde (2 mmoL). It was recrystallized from DMSO as white crystals. Recrystallization from ethanol as white crystals.

Bis 2-(1,3,4-thiadiazol-2,5-diylthio)-N'-((1-phenyl-1H-pyrazolo[4,3-b]quinoxalin-3-yl)methylene)aceto-hydrazide **6**. It was obtained from 2,5-bis(mercapto-acetohydrazide)-1,3,4-thiadiazole **3** (2 mmoL) and 1-phenyl-1*H*-pyrazole [4,3-*b*] quinoxaline-3-carbaldehyde (2 mmoL). Recrystallization from DMSO as orange crystals.

Materials (Antiproliferative screening)

Mammalian cell lines: HepG-2 (hepatocellular carcinoma), HCT-116 (colon carcinoma) and MCF-7 cells (human Breast cancer cell line), were obtained from VACSERA Tissue Culture Unit.

Chemicals Used: Dimethyl sulfoxide (DMSO), crystal violet and trypan blue dye were purchased from Sigma (St. Louis, Mo., USA). Fetal Bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza.

Crystal violet stain (1%): It composed of 0.5% (w/v) crystal violet and 50% methanol then made up to volume with ddH_2O and filtered through a Whatmann No.1 filter paper.

Cell line propagation:

The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin. All cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were subcultured two times a week.

Cytotoxicity evaluation using viability assay:

For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1×10^4 cells per well in 100µl of growth medium. Fresh medium containing different concentrations of the test sample was added after 24 h of seeding. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37ºC in a humidified incubator with 5% CO_2 for a period of 24 h. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. After incubation of the cells for at 37°C, for 24 h, the viable cells yield was determined by a colorimetric method. In brief, after the end of the incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 minutes. The stain was removed and the plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on Microplate reader (TECAN, Inc.), using a test wavelength of 490 nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated[53,54]. The optical density was measured with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA).

III. Result

A. Chemical analysis.

Table no 1: Mass, IR, 1H NMR spectral data of compounds **4-6**.

Comp. no.	EI-MS: *m/z*	IR(γ, cm-1)	^{1}H NMR (δ, ppm) (DMSO-d_6)	^{13}C NMR (δ, ppm) (DMSO-d_6)
4	*m/z* 624 [M^+]	3446 (NH), 1714 (CO-ester), 1679 (CO-amide), 1602 (C=N)	δ: 1.253-1.283 (2t, 6H, $2CH_{3(ester)}$), 2.490-2.586 (2ss, 6H, $2CH_{3(furan)}$), 4.200-4.259 (m, 4H, $2CH_{2(ester)}$), 4.115-4.523 (2d, 4H, $2SCH_2$), 7.079, 7.097 (2s, 2H, $2CH_{(furan)}$), 7.836, 7.992 (2s, 2H, 2CH=N), 11.722, 11.799 (2s, 2H, 2NH, D_2O-exchangeable).	δ: 13.689,13737 and 14.166 ($2CH_{3(ester)}$ and $2CH_{3(furan)}$), 36.066 and 36.123 ($2CH_{3(ester)}$), 36.267 and 36.314 ($2SCH_2$), 114.023, 114.055, 114.681, 115.024, 133.338, 136.056, 160.599 and 160.751 (8 carbons of two furan rings), 160.599, 160.751, 162.544, 163.098, 164.853, 165.291 (6 carbons of CH=N), 168.000 (CO-NH), 192.211 (CO-ester)
5	*m/z* 604 [M^+]	3442 (NH), 1655 (C=O; amide), 1566 (C=N)	δ: 4.184-4.594 (2ss, 4H, $2CH_2$), 7.422-7.462 (dd, 2H, Ar-$H_{(para)}$), 7.552-7.593 (dd, 4H, Ar-$H_{(meta)}$), 8.007-8.021 (dd, 4H, Ar-$H_{(ortho)}$), 8.188, 8.375 (2s, 2H, 2 CH=N), 8.412, 8.463 (2s, 2H, 4 CH=$N_{(triazole)}$),11.931, 12.022 (2s, 2H, 2NH, D_2O exchangeable).	δ: 35.751 and 36.267 ($2SCH_2$), 118.458, 118.496, 128.130, 128.132, 129.809, 134.282, 134.492 and 134.721 (10 carbons of two phenyl rings), 145.194 (2 CH=$N_{(open\ chain)}$), 162.800, 163.000, 163.260, 164.980, 164.929 and 165.220 (4CH=$N_{(two\ triazole\ rings)}$, 168.220 (2CO-NH).
6	*m/z* 806 [M^+]	3438 (NH), 1665 (C=O; amide), 1600 (C=N)	δ: 4.278,4.457,4.625, 4.812 (4s, 4H, $2SCH_2$), 7.374-7.478 (m, 2H, 2*Ar-*$H_{(a)}$), 7.568-7.645 (m, 4H, 4*Ar-*$H_{(b)}$), 7.765-7.877 (m, 4H, 4*Ar-*$H_{(d)}$), 7.915-7.950 (m, 2H, 2CH=N, 4*Ar-*$H_{(e)}$), 8.186-8.319 (m, 4H, 4*Ar-*$H_{(c)}$), 12.110, 12.730 (2s, 2H, 2NH, D_2O exchangeable).	----------

Table no 2: Physical constants of *bis carbohydrazono-1,3,4-thiadiazoles* **4-6**.

Compound No.	Yield (%)	M.p. (°C)	Mol. Form. (Mol. Wt.)	Rf	Microanalysis (expected/found)				
					C	H	N	O	S
4	93	168-170	$C_{24}H_{28}N_6O_8S_3$ 624	0.58 (EA: H; 5:1; V/V)	46.14 46.04	4.52 4.42	13.45 13.50	20.49 20.58	15.40 15.29
5	95	188-190	$C_{24}H_{20}N_{12}O_2S_3$ 604	0.71 (EA: H; 5:1; V/V)	47.67 47.76	3.33 3.24	27.80 27.91	5.29 5.35	15.91 15.84
6	99	192	$C_{38}H_{26}N_{14}O_2S_3$ 806	0.52 (C: M; 15:1; V/V)	56.56 56.66	3.25 3.18	24.30 24.21	3.97 4.10	11.92 11.81

Table no 3: Effect of standard compound (Vinblastine Sulfate) on cell viability using cytotoxic assay.

Conc. (µg/mL)	MCF-7				HCT-116				HepG-2			
	Viability % (3 Replicates)				Viability % (3 Replicates)				Viability % (3 Replicates)			
	1 st	2 nd	3 rd	**Mean**	1 st	2 nd	3 rd	**Mean**	1 st	2 nd	3 rd	**Mean**
500	4.85	5.17	4.59	**4.87**	2.83	3.45	2.36	**2.88**	2.98	3.21	2.64	**2.94**
250	6.78	9.06	8.12	**7.99**	5.36	6.74	5.89	**6.00**	6.53	5.49	5.49	**5.84**
125	13.91	15.43	12.85	**14.06**	9.25	12.93	10.41	**10.86**	9.74	9.86	7.57	**9.06**
62.5	19.87	21.62	18.06	**19.85**	13.88	16.42	14.97	**15.09**	12.81	13.92	12.4	**13.04**
31.25	25.14	23.95	21.92	**23.67**	18.6	23.65	20.43	**20.89**	20.36	21.85	19.62	**20.61**
15.6	31.67	28.74	31.67	**30.69**	26.78	30.51	28.19	**28.49**	31.52	29.68	27.44	**29.55**
7.8	36.85	39.16	40.83	**38.95**	33.67	38.94	35.46	**36.02**	38.06	38.06	35.81	**37.31**
3.9	42.79	47.82	51.29	**47.30**	40.89	46.06	41.37	**42.77**	46.23	44.52	43.97	**44.91**
2	58.14	60.67	62.38	**60.40**	48.24	54.93	50.68	**51.28**	57.32	53.17	55.24	**55.24**
1	69.43	74.18	71.54	**71.72**	57.12	63.78	59.45	**60.12**	63.91	63.91	65.46	**64.43**
0	100	100	100	100	100	100	100	100	100	100	100	100

Table no 4: Effect of different concentrations of compound **4** on cell viability using cytotoxic assay.

Conc. (µg/mL)	HepG-2			HCT-116			MCF-7		
	Viability %	Inhibitory %	S.D. (±)	Viability %	Inhibitory %	S.D. (±)	Viability %	Inhibitory %	S.D. (±)
500	**20.97**	79.03	1.49	**28.72**	71.28	2.69	**28.51**	71.49	2.43
250	**46.93**	53.07	3.15	**49.21**	50.79	3.45	**48.67**	51.33	2.71
125	**78.08**	21.92	2.36	**86.49**	13.51	1.87	**83.29**	16.71	3.85
62.5	**95.66**	4.34	0.82	**98.02**	1.98	0.84	**95.41**	4.59	1.07
31.25	**99.42**	0.58	0.56	**100**	0		**99.26**	0.74	0.72
15.6	**100**	0		**100**	0		**100**	0	

7.8	**100**	0		**100**	0		**100**	0	
3.9	**100**	0		**100**	0		**100**	0	
0	**100**	0		**100**	0		**100**	0	

Table no 5: Effect of different concentrations of compound **5** on cell viability using cytotoxic assay.

Conc. (µg/mL)	HepG-2			HCT-116			MCF-7		
	Viability %	Inhibitory %	S.D. (±)	Viability %	Inhibitory %	S.D. (±)	Viability %	Inhibitory %	S.D. (±)
500	**8.49**	91.51	1.08	**10.65**	89.35	1.34	**14.53**	85.47	1.39
250	**21.32**	78.68	1.74	**26.84**	73.16	0.89	**31.78**	68.22	0.64
125	**34.65**	65.35	2.83	**38.73**	61.27	2.91	**46.21**	53.79	2.37
62.5	**60.91**	39.09	3.17	**71.46**	28.54	2.86	**81.92**	18.08	3.46
31.25	**78.47**	21.53	1.45	**86.79**	13.21	1.93	**96.84**	3.16	1.28
15.6	**90.64**	9.36	0.42	**98.06**	1.94	0.82	**100**	0	
7.8	**97.21**	2.79	0.79	**100**	0		**100**	0	
3.9	**100**	0		**100**	0		**100**	0	
0	**100**	0		**100**	0		**100**	0	

Table no 6: Effect of different concentrations of compound **6** on cell viability using cytotoxic assay.

Conc. (µg/mL)	HepG-2			HCT-116			MCF-7		
	Viability %	Inhibitory %	S.D. (±)	Viability %	Inhibitory %	S.D. (±)	Viability %	Inhibitory %	S.D. (±)
500	**11.36**	88.64	0.72	**17.48**	82.52	1.86	**19.74**	80.26	2.42
250	**26.74**	73.26	1.84	**31.65**	68.35	1.93	**33.89**	66.11	3.17
125	**39.82**	60.18	2.67	**45.06**	54.94	3.18	**46.28**	53.72	3.46
62.5	**54.43**	45.57	3.65	**76.84**	23.16	2.98	**80.67**	19.33	2.91
31.25	**70.64**	29.36	1.42	**90.63**	9.37	1.05	**94.23**	5.77	1.46
15.6	**88.59**	11.41	0.73	**97.65**	2.35	0.93	**98.71**	1.29	0.65
7.8	**98.04**	1.96	0.62	**100**	0		**100**	0	
3.9	**100**	0		**100**	0		**100**	0	
0	**100**	0		**100**	0		**100**	0	

Table no 7: IC_{50} of Standard and Compounds **4-6** on cell viability using cytotoxic assay compared to standard.

	IC_{50} (µg/mL)		
	HepG-2	**HCT-116**	**MCF-7**
Standard	3.02 ± 0.26	2.35 ± 0.39	3.57 ±0.45
Cpd. **4**	238± 6.9	247 ± 8.1	245 ± 7.8
Cpd. **5**	88.5± 3.1	103 ± 3.4	118 ± 5.3
Cpd. **6**	81.5± 3.7	115± 5.4	118± 5.7

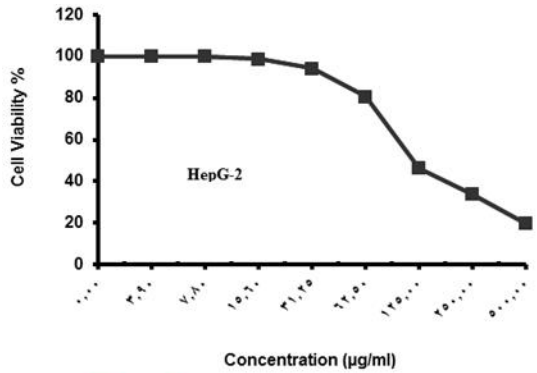


Figure 1: Viability activity against HepG-2 of compound **4**

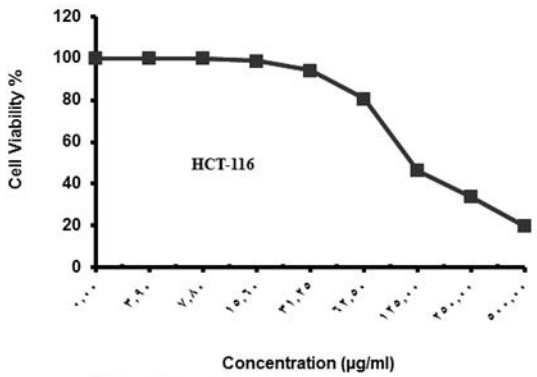


Figure 2: Viability activity against HCT-116 of compound **4**

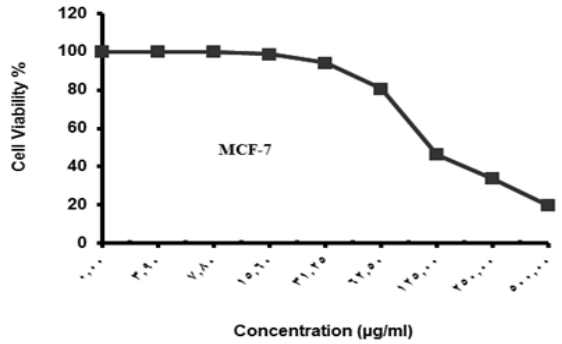


Figure 3: Viability activity against MCF-7 of compound **4**

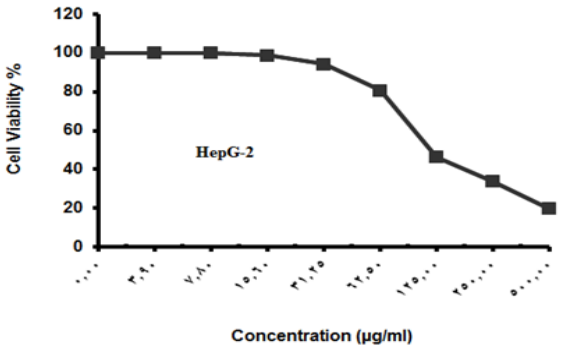


Figure 4: Viability activity against HepG-2 of compound **5**

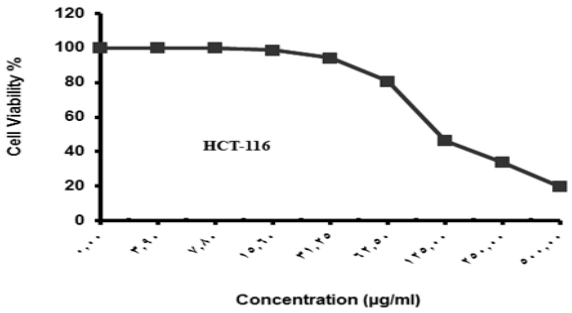


Figure 5: Viability activity against HCT-116 of compound **5**

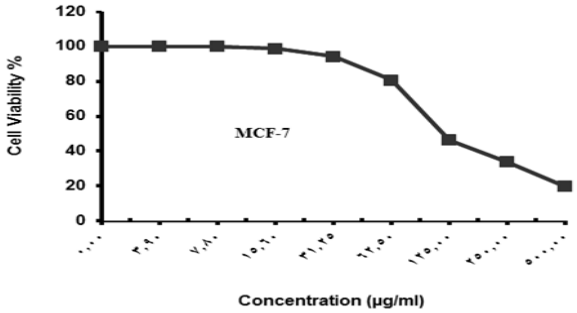


Figure 6: Viability activity against MCF-7 of compound **5**

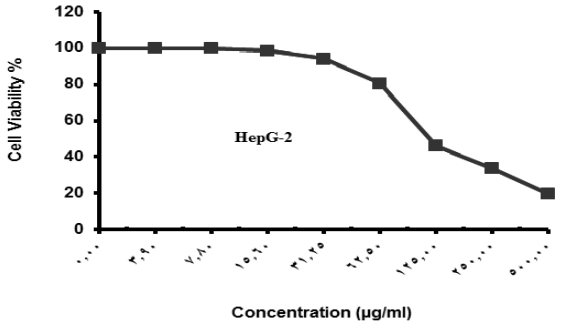


Figure 7: Viability activity against HepG-2 of compound 6

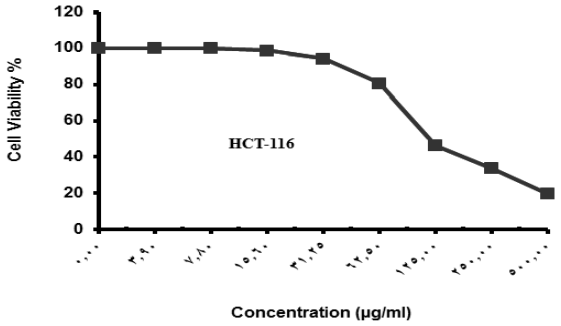


Figure 8: Viability activity against HCT-116 of compound 6

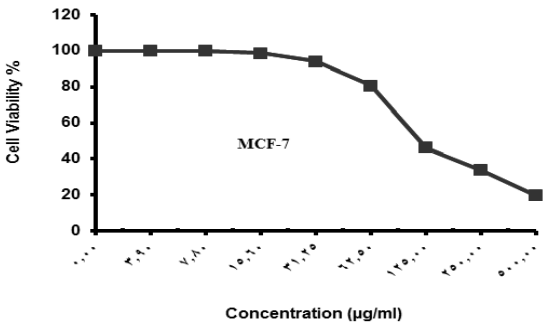
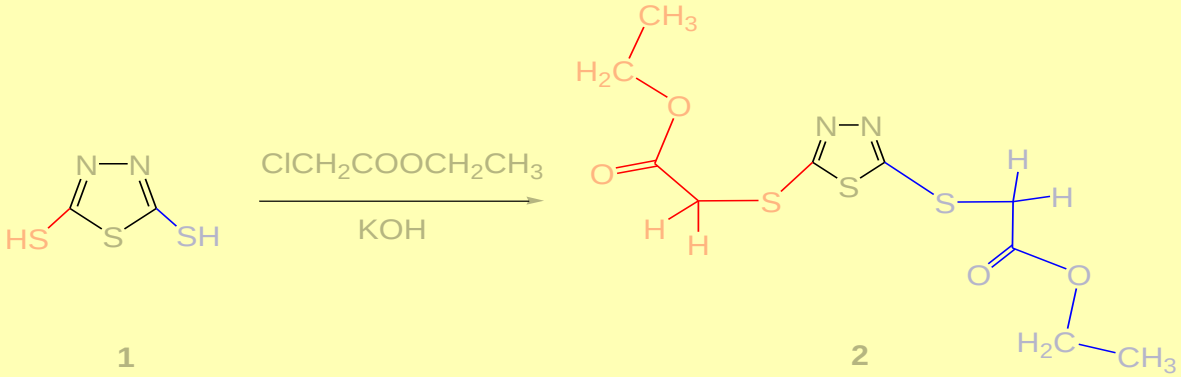


Figure 9: Viability activity against MCF-7 of compound 6

IV. Discussion

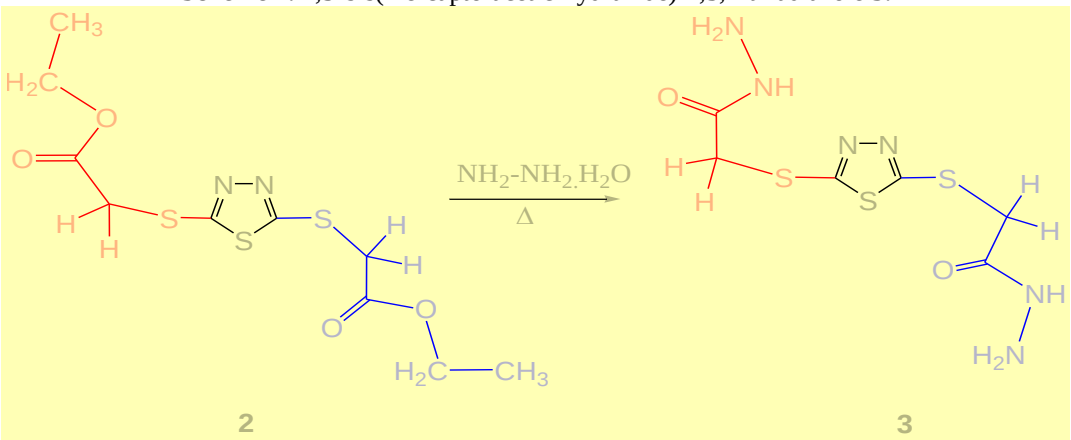
Condensation of 2,5-dimercapto-1,3,4-thiadiazole **1**[52] with ethyl chloroacetate in basic medium, resulted in bis ethyl 2-(1,3,4-thiadiazol-2,5-diylthio) acetate **2**[52],Scheme 1.

Scheme 1. Synthesis of bis ethyl 2-(1,3,4-thiadiazol-2,5-diylthio) acetate **2.**



Treatment of diacetate derivative **2** with hydrazine hydrate (80%), resulted in the corresponding 2,5-bis(mercapto-acetic-hydrazide)-1,3,4-thiadiazole **3**[52], Scheme 2.

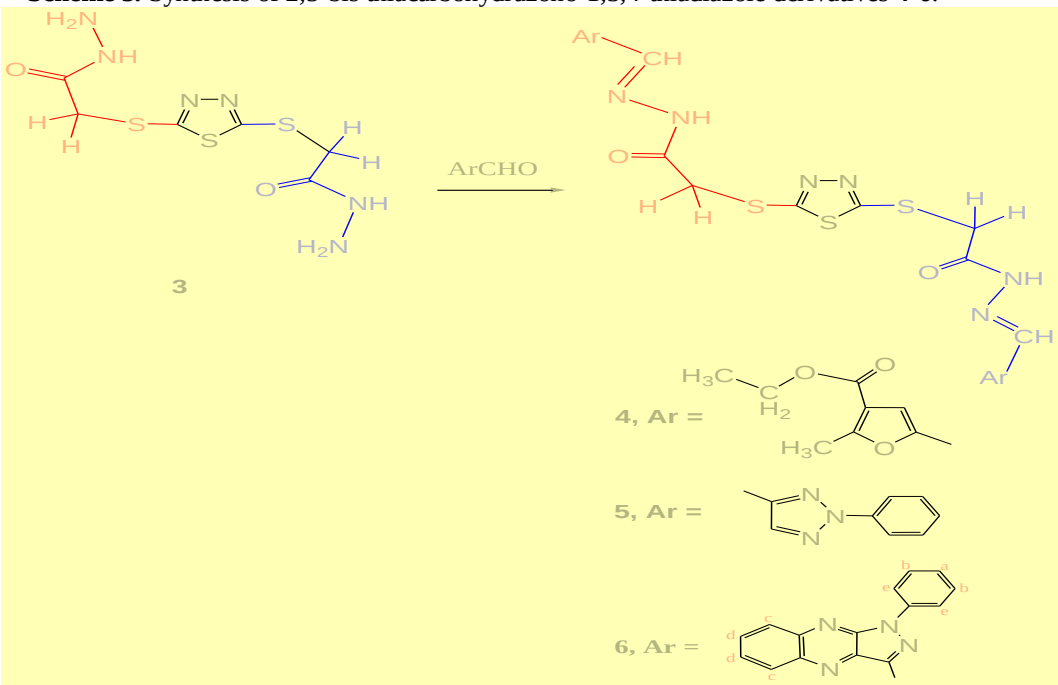
Scheme 2. 2,5-bis(mercapto-acetic-hydrazide)-1,3,4-thiadiazole **3.**



Condensation of bis carbohydrazide derivative **3** with 5-methyl-4-propionylfuran-2-carbaldehyde, 2-phenyl-2*H*-1,2,3-triazole-4-carbaldehyde and 1-phenyl-1*H*-pyrazole [4,3-*b*] quinoxaline-3-carbaldehyde, afforded the corresponding 2,5-bis thiacarbohydrazono-1,3,4-thiadiazole derivatives **4-6**, respectively, Scheme 3.

The structures of derivatives **4-6** were confirmed by IR, ^{1}HNMR, ^{13}CNMR and mass spectral data. ^{1}H NMR spectrum (DMSO-d_6) of compound **4** showed two triplets nearly at the same region at δ 1.283-1.253 ppm corresponding to the six protons of the two methyl ester groups, followed by two splitted singlets at 2.586-2.490 ppm for the six protons of the two methyl groups at position-5 at the two furan rings. The four protons of the two methylene ester groups were showed as two quartets nearly at the same region δ 4.259-4.200 ppm, followed by two doublets at δ 4.523-4.115 ppm due to the four protons of the other methylene groups. Two singlets were shown at δ 7.097 and 7.079 ppm for the two protons at the position-3 of the furan rings, followed by other two singlets at δ 7.992 and 7.836 ppm corresponding to the two imine protons (CH=N). The two NH protons (D_2O exchangeable) were resonated as two singlets at δ 11.799 and 11.792; see (Table 1 and scheme 3).

Scheme 3. Synthesis of 2,5-bis thiacarbohydrazono-1,3,4-thiadiazole derivatives **4-6**.



In vitro antiproliferative activity screening (cytotoxicity against three cancer cell lines):

Different concentrations (500-3.9 µg/mL) of the examined compounds **4-6** were used to screen their cytotoxicity against Human Hepatocellular Liver Carcinoma Cells (HepG-2), Human Colon Carcinoma Cells (HCT-116) and Human Breast Adrenocarcinoma Cells (MCF-7) by viability assay in terms of IC_{50}. Cytotoxic effect of these compounds on cell viability of cancer cell lines was observed as shown in Tables (4-7) and Figures (1-9). The relationships between the structures of synthesized compounds **4-6** and their antiproliferative activity were investigated from their IC_{50} values, where the 1,3,4-thiadiazole-based heterocycles **5** and **6** have excellent cell growth inhibitory effects on HepG-2, HCT-116 and MCF-7 compared to derivative **4**, may be due to the presence of aliphatic part ($CO_2CH_2CH_3$) as well as lower nitrogen content in derivative **4** as compared to derivatives **5** and **6**, see Scheme 3 and Tables (1&7).

V. Conclusion

In conclusion, new 1,3,4-Thiadiazole-based heterocycles have been prepared. Their physical and chemical properties were studied, indeed these compounds showed antiproliferative activities. The relationships between the structures of synthesized compounds and their antiproliferative activity were investigated from their IC_{50} values.

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