

Synthesis and Evaluation of Antiproliferative activity of 1, 2, 4-Triazole Derivatives Against EAC Bearing Mice Model

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ABSTRACT

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A series of potential bioactive compounds, 4-Amino-5-mercapto-3-(4-chlorophenyl)-1, 2, 4-triazole (1d), 4-Amino-5-mercapto-3-(4-nitrophenyl)-1, 2, 4-triazole (2d), 4-Amino-5-mercapto-3-(2, 4-dichlorophenyl)-1, 2, 4-triazole (3d), 4-Amino-5-mercapto-3-(2-chlorophenyl)-1, 2, 4-triazole (4d), 4-Amino-5-mercapto-3-(2-methylphenyl)-1, 2, 4-triazole (5d), 4-Amino-5-mercapto-3-(4-methoxyphenyl)-1, 2, 4-triazole (6d) were synthesized according to the literature methods. The synthesized compounds were characterized by FT-IR, ¹H NMR spectroscopy, C H N analysis and anticancer activity was evaluated.

Male Swiss albino mice has been used as test animal. Tumor cells used for anticancer activity were EAC (Ehrlich Ascites Carcinoma). Compounds were given at a dose of 25 mg/kg body weight intraperitoneally. Compounds treated (III-VIII) groups were found to reduce tumor volume, viable cell count and increase the tumor weight (%) inhibition, ascites cells (%) inhibition and non-viable cell count and increase in life span (%ILS). All the compounds exhibited the significant (P < 0.01) anticancer activity compared to control and the compound 4d is found to be the most potent one. The standard drug was used as 5-Fluorouracil (20mg/kg, body weight).

Keywords: 1, 2, 4-triazole, anticancer activity, tumor cell count, tumor weight inhibition.

INTRODUCTION

Cancer is a killer disease, more dangerous than any other disease except AIDS. It is a second most occurring disease after cardiovascular disease. It is a life threatening disease and remains a major health problem around the globe¹. The substances containing a five member heterocyclic base are important targets in chemical synthesis because of their pronounced biological activities². The chemistry of N-bridged heterocycles derived from 1, 2, 4-triazole has received considerable attention in recent years due to their usefulness in different areas of biological activities and as industrial intermediates³. A large number of heterocyclic compounds containing the 1, 2, 4-triazole ring and N-C-S linkage of triazole especially responsible for biological activities² i.e. antifungal³, anti-tubercular⁴, anti-inflammatory⁵, anticonvulsant⁶, antibacterial⁷, antiviral⁸ and antitumor⁹⁻¹¹ etc.

There are also some known drugs containing 1, 2, 4-triazole moiety, e.g. Triazolam, Alprazolam, Etizolam, Furacylin, Ribavirin, Hexaconazole, Triadimefon, Mycobutanil,

Rizatriptan, Propiconazole, Fluotrimazole which are extensively used for various disorders. In the present investigation we reported some 4-Amino-5-mercapto 3-(substituted)-1, 2, 4-triazole derivatives (1d-6d) and screened them for anticancer properties.

GENERAL CHEMISTRY

FT-IR spectra were recorded on a Bruker Vertex 79 FT-IR spectrophotometer, using KBr pellet technique. The structures of all the compounds were confirmed by ¹H NMR spectra with Bruker DPX 300 MHz Instrument (DMSO-d₆, CDCl₃ and TMS) and elemental analysis using C H N analyzer 2400 ser II (Perkin Elmer). Melting points were determined with Veego melting point apparatus. All solvents and reagents were purchased from M/S Merck and M/S SD Fine Chemical and were used, when necessary, after purification.

SYNTHESIS

Aromatic acid (1a-6a) was esterified with methanol using sulfuric acid according to the literature methods¹² in Scheme 1 and the resulting compounds (1b-6b) were refluxed with hydrazine hydrate in ethanol to give aroyl hydrazine (1c-6c). Aroyl hydrazines were refluxed with NH₂NH₂·H₂O, CS₂ and KOH in ethanol to get cyclized to form aryl derivatives of 4-Amino-5-mercapto-3-(substituted)-1, 2, 4-triazole (1d-6d)

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in good yield. The structure of these compounds have been elucidated by spectral and elemental (FT-IR, ^1H NMR and C H N analysis) analysis¹².

METHODOLOGY AND EXPERIMENTAL WORK

General Scheme of Synthesis

Synthesis of substituted methyl benzoate derivative (1b-6b):

In a clean dried 250 ml round bottomed flask, methanol (60 ml, 1.50 mol) and conc. sulfuric acid (H_2SO_4) (2-3 ml) were taken. Then benzoic acid derivative (0.06 mol) was added into the flask and refluxed for 6hrs. The flask was then cooled to room temperature and the contents were concentrated by rotary evaporator and dried at room temperature and recrystallized from ethanol.

Synthesis of substituted benzoic acid hydrazide derivative (1c-6c):

Hydrazine hydrate (6.00 gm, 0.12 mol) was placed in a small round bottomed flask fitted with reflux condenser and then methyl benzoate (0.04 mol) was added and gently heated under refluxed for 10 minutes. Sufficient quantity of absolute alcohol was added through the condenser to get a clear solution (about 10 ml). Then the reaction mixture was further refluxed for 4 hrs. The reaction mixture was concentrated and cooled to room temperature. The crystals of acid hydrazide were dried and recrystallized from ethanol.

Synthesis of 4-Amino -5-mercapto-3-(substituted phenyl)-1, 2, 4-triazole (1d-6d):

The acid hydrazide (0.01 mol) was added to absolute alcohol containing potassium hydroxide (KOH) (1.6 gm) at room temperature. Carbon disulfide (CS_2) (1.8 ml) was added and the mixture stirred at room temperature for 10 hrs. The mixture was diluted with ether and stirred for a further 1 hr. The potassium salts was used for the next step without further purification. Hydrazine hydrate (99%) (0.02 mol 1.00 gm) was gradually added to the above potassium salt, then dissolved in water (20 ml) with stirring and the mixture was refluxed gently for 3 hrs during which hydrogen sulphide evolved and the color of the reaction mixture change to a dark green color. It was then cooled to 5°C and acidified. A solid separated out and which was filtered and recrystallized from ethanol to make triazole.

Spectral data of the synthesized compounds:

4-Amino- 5-mercapto- 3-(4-chlorophenyl)-1, 2, 4-triazole (1d)

Melting point - $220-222^\circ\text{C}$. R_f value – 0.54, Yield: 80.01%, Chemical formula: $\text{C}_8\text{H}_7\text{N}_4\text{ClS}$. Chloroform: Methanol = 8:2.

FT-IR (V_{\max} cm^{-1}): 3116(NH_2), 2942 (ArC-H), 723 (Ar-C-Cl), 1611 (C=N), 1406 (C-N), 1466(C=C), 1179(C-C), 1281 (C=S). ^1H NMR (300 MHz , $\text{DMSO}-d_6$ p p m): 7.50 (d, $J=9\text{Hz}$, 2H, 2', 6' Ar-H); 8.12 (d, $J=9\text{Hz}$, 2H, 3' 5' Ar-H); 3.42 (s, 1H, SH.); 2.72(s, 2H, NH_2). Anal. Calcd. For $\text{C}_8\text{H}_7\text{N}_4\text{ClS}$. C, 42.38; H, 53.09; N, 24.72. Found: C, 42.38; H, 53.11; N, 24.52.

4-Amino- 5-mercapto- 3-(4-nitrophenyl)-1, 2, 4-triazole (2d)

Melting Point – $199-201^\circ\text{C}$. R_f value – 0.4, Yield: 68.35%, Chemical formula: $\text{C}_8\text{H}_7\text{O}_2\text{N}_5\text{S}$. Chloroform: Methanol = 8:2. FT-IR (V_{\max} cm^{-1}): 3381(NH_2), 2991 (ArC-H), 1517(Ar-C- NO_2), 1685 (C=N), 1422 (C-N), 1604(C=C), 1176(C-C), 1283(C=S). ^1H NMR (300 MHz , $\text{DMSO}-d_6$ p p m): 6.64 (d, $J=9\text{Hz}$, 2H, 2', 6' Ar-H); 7.53 (d, $J=9\text{Hz}$, 2H, 3' 5' Ar-H); 2.50 (s, 2H, NH_2); 3.16 (s, 1H, SH). Anal. Calcd. For $\text{C}_8\text{H}_7\text{O}_2\text{N}_5\text{S}$. C, 49.23; H, 3.58; N, 14.35. Found: C, 49.38; H, 3.69; N, 14.52.

4-Amino- 5-mercapto- 3-(2, 4-dichlorophenyl)-1, 2, 4-triazole (3d)

Melting point – $212-214^\circ\text{C}$; R_f value-0.75; Yield: 78.25%, Chemical formula $\text{C}_8\text{H}_6\text{N}_4\text{Cl}_2\text{S}$; Ethanol: Benzene = 1: 4; FT-IR (V_{\max} cm^{-1}): 3306 (NH_2), 3087 (ArC-H), 827 (Ar-C-Cl), 1653 (C=N), 1467 (C-N), 1588(C=C), 1105(C-C), 1310(C=S). ^1H NMR (300 MHz , $\text{DMSO}-d_6$ p p m): δ 2.72 (s, 2H, NH_2); δ 3.41 (s, H, SH); δ 8.00 (s, 1H, Ar-H). δ 8.17 (s, 1H, Ar-H). δ 7.75 (s, 1H, Ar-H). Anal. Calcd. For $\text{C}_8\text{H}_6\text{N}_4\text{Cl}_2\text{S}$. C, 36.78; H, 2.29; N, 21.45. Found: C, 36.88; H, 2.53; N, 21.55.

4-Amino- 5-mercapto- 3-(2-chlorophenyl)-1, 2, 4-triazole (4d)

Melting point $-193-195^\circ\text{C}$. R_f value-0.82, Yield: 70.198%, Chemical formula: $\text{C}_8\text{H}_7\text{N}_4\text{ClS}$. Chloroform: Methanol = 4: 1, FT-IR (V_{\max} cm^{-1}): 3287(NH_2), 3061 (ArC-H), 718 (Ar-C-Cl), 1604 (C=N), 1464(C-N), 1272(C-C), 1267 (C=S). ^1H NMR (300 MHz , $\text{DMSO}-d_6$ p p m): δ 2.76 (s, 2H, NH_2); δ 3.16 (s, 1H, SH); δ 7.06-7.92 (m, 4H, Ar-H). Anal. Calcd. For $\text{C}_8\text{H}_7\text{N}_4\text{ClS}$. C, 42.38; H, 3.09; N, 24.72. Found: C, 42.42; H, 3.13; N, 24.85.

4-Amino- 5-mercapto- 3-(2-methylphenyl)-1, 2, 4-triazole (5d)

Melting point - $180-183^\circ\text{C}$. R_f value – 0.84; Yield: 70.87%, Chemical formula: $\text{C}_9\text{H}_{10}\text{N}_4\text{S}$. Chloroform: Methanol = 4: 1. IR (V_{\max} cm^{-1}): 3109 (NH_2), 2950 (ArC-H), 1606 (C=N), 2755 (ArC- CH_3), 1448 (C-N), 1577(C=C), 1194(C-C), 1294(C=S). ^1H NMR (300 MHz , $\text{DMSO}-d_6$ p p m): δ 7.16-7.88

(m, 4H, ArH); δ 3.10 (s, 1H, SH); δ 2.26 (s, 2H, NH₂); δ 2.53 (d, 3H, CH₃). Anal. Calcd. For C₉H₁₀N₄S. C, 52.42; H, 4.85; N, 27.18. Found: C, 52.52; H, 4.93; N, 27.15.

4-Amino- 5-mercaptop- 3-(4-methoxyphenyl)-1, 2, 4-triazole (6d)

Melting point - 179°C. R_f value – 0.81, Yield: 62.61%, Chemical formula: C₉H₁₀ON₄S. Chloroform: Methanol = 4: 1. FT-IR (V_{max} cm⁻¹): 3158 (NH₂), 2937 (ArC-H), 1573 (C=N), 1609 (ArC-OCH₃), 1492 (C-N), 1352 (C-C), 1294(C=S). ¹H NMR (300 MHz, DMSO d₆ p p m): δ 7.43(d, J= 9Hz, 2H, 2', 6', Ar-H), δ 8.17 (d, J= 9Hz, 2H, 3' 5', Ar-H), δ 2.272(s, 2H, NH₂), δ 6.35 (s, 3H, OCH₃), δ 3.34 (s, 1H, SH). Anal. Calcd. For C₉H₁₀ON₄S. C, 48.64; H, 4.58; N, 25.22. Found: C, 48.70; H, 4.61; N, 25.15.

EXPERIMENTAL PROCEDURE

Evaluation of anticancer activity:

Male Swiss albino mice of 10 weeks old with an average body weight of 18 to 20 g were used. All mice are kept on basal metabolic diet with water *ad libitum*. Male Swiss albino mice were divided into 9 groups (n = 6). EAC cells were collected from the donor mice and are suspended in sterile isotonic solution (0.9% w/v NaCl). The numbers of tumor cells per ml of this suspension are counted under microscope with the help of haemocytometer. All the groups were treated with EAC cells (0.2 ml of 2x10⁶ cells/mouse) intraperitoneally except the normal control group (I). This was taken as day zero. After 24 hrs of tumor inoculation the synthesized compounds (**1d-6d**, 25mg/kg body weight/day) and the standard drug 5-Fluorouracil (20mg/kg body weight/day) were administered in groups (III-VIII) and (IX) respectively for 7 days. On the 9th day food and water were withheld 18 hr before starting the testing operation. The weights of all the animals were recorded before they were sacrificed. The animals were anaesthetized and dissected to expose the peritoneal cavity and by a syringe the ascetic fluid was withdrawn to a suitable volume, collected in sterile ice-cold saline and preserved in ice bath. The total number of living cells/ml in the peritoneal fluid of the 6 mice in a group is calculated. The fluid is sucked by adsorbent cotton. The weight of the 6 mice after sacrifice was recorded. After sacrificing the animals, blood was collected to evaluate the hematological parameters¹²⁻¹³.

The evaluation of the test drug is made by comparing the cell count of the test with that of the control. The percentage inhibition of cell count is obtained by following expression:

$$\text{Percentage inhibition of ascitic cells (\%TCI)} = (1 - T/C) \times 100$$

Where T is the average number of ascitic cells /ml in test

animals, C is the average number of the ascetic cells /ml in control animals.

The anti-tumor activity of the compounds were measured in EAC animals with respect to the following parameters such as:

Tumor Weight

The tumor weight¹² was calculated according to Ghosh *et al.*, 2010.

Tumor Cell Count

The tumor cells counts¹² was calculated according to Ghosh *et al.*, 2010.

Hematological Parameters

The blood was collected by retro-orbital puncture under ether anesthesia and subjected to the estimation of hematological parameters like hemoglobin content (Hb), red blood cells (RBC) count, and white blood cells (WBC) count¹³.

Survival Time

The effect of the compound on life span was measured by calculating MST as follows, MST = (day of first death + day of last death of animal)/2. The percentage increase in life span (%ILS) was calculated using the following formula: %ILS = (Mean survival of treated group- Mean survival of control group)/ Mean survival of control group \times 100¹⁴. An enhancement of life span by 20% or more was considered as effective response¹⁴.

All procedures described were reviewed and approved by the University Animals Ethical Committee. The recommendations of Jadavpur University Institutional Animal Ethics Committee [Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA registration. no. 0367/01/C/CPCSEA) India for the care and use of laboratory animals were strictly followed throughout the study and these were in accordance with the NIH (USA) guidelines].

RESULT AND DISCUSSION

In this study, all the synthesized compounds were investigated for anticancer activity on EAC bearing mice. In case of tumor growth response study, triazole derivatives (**1d-6d**) significantly reduced tumor volume and viable cell count as compared to those of EAC control mice, while nonviable cell count, increase in life span (%ILS), tumor weight (%) inhibition, ascites cells (%) inhibition was found to be increased significantly in the treated groups. These results were showed in Table.1, 2 and 3.

The reliable criteria for judging the value of any anticancer

Table 1: Anticancer activity of triazole derivatives against % tumor weight inhibition (% TWI) and % tumor count inhibition (%TCI) of EAC bearing mice

Group	Compound	Avg. tumor weight (g)	% TWI	Avg. cell count ($\times 10^6$ cells/ml)	%TCI
I	Normal control	-	-	-	-
II	Induce control	3.64 \pm 0.03	0.00	206.03 \pm 0.5	0.00
III	1d	2.15 \pm 0.12*	40.93	109 \pm 0.13*	46.72
IV	2d	2.52 \pm 0.3*	30.76	133.75 \pm 0.15*	35.07
V	3d	1.80 \pm 0.11*	50.54	83.75 \pm 0.21*	59.34
VI	4d	1.70 \pm 0.05*	53.29	75.03 \pm 0.15*	63.59
VII	5d	2.25 \pm 0.15*	38.18	143.25 \pm 0.09*	30.46
VIII	6d	2.80 \pm 0.03*	23.07	162.25 \pm 0.12*	21.23
IX	5 FU	-	100	-	100

Value are Mean \pm SEM. n=6 animal in each group. Experimental groups were compared with Induce control; *P< 0.01

Table 2: Anticancer activity of triazole derivatives on RBC, WBC, tumor volume, % viable and % non viable tumor cell count of EAC bearing mice.

Group	Compounds	RBC (10^6 cells)	WBC (10^3 cells)	Tumor volume (ml)	% of Viable cell	% of Non-viable cell
I	Normal control	11.84 \pm 0.33	2450.10 \pm 0.25	-	-	-
II	Induce control	2.87 \pm 0.13	4500.12 \pm 0.23	3.85 \pm 0.05	190.77 \pm 0.95	15.26 \pm 0.15
III	1d	6.12 \pm 0.09*	3100.13 \pm 0.12*	0.81 \pm 0.03*	80.12 \pm 0.09*	29.60 \pm 0.12*
IV	2d	5.76 \pm 0.11*	4100.14 \pm 0.11*	0.87 \pm 0.05*	110.75 \pm 0.23*	23.01 \pm 0.15*
V	3d	6.23 \pm 0.05*	5100.05 \pm 0.05*	0.56 \pm 0.07*	45.60 \pm 0.12*	38.15 \pm 0.13*
VI	4d	7.02 \pm 0.21*	3500.12 \pm 0.013*	0.45 \pm 0.05*	29.12 \pm 0.35*	45.91 \pm 0.10*
VII	5d	4.89 \pm 0.18*	2400.21 \pm 0.10*	0.98 \pm 0.09*	121.15 \pm 0.23*	21.10 \pm 0.25*
VIII	6d	3.55 \pm 0.07*	6700.10 \pm 0.09*	1.05 \pm 0.05*	142.05 \pm 0.07*	20.20 \pm 0.41*
IX	5 FU	10.32 \pm 0.15*	2500.11 \pm 0.06*	-	-	-

Value are Mean \pm SEM. n=6 animal in each group. Experimental groups were compared with Induce control; *P< 0.01

Table 3: Anticancer activity of triazole derivatives on Hemoglobin (HB), Mean survival time (MST), Increase in life span (%ILS).

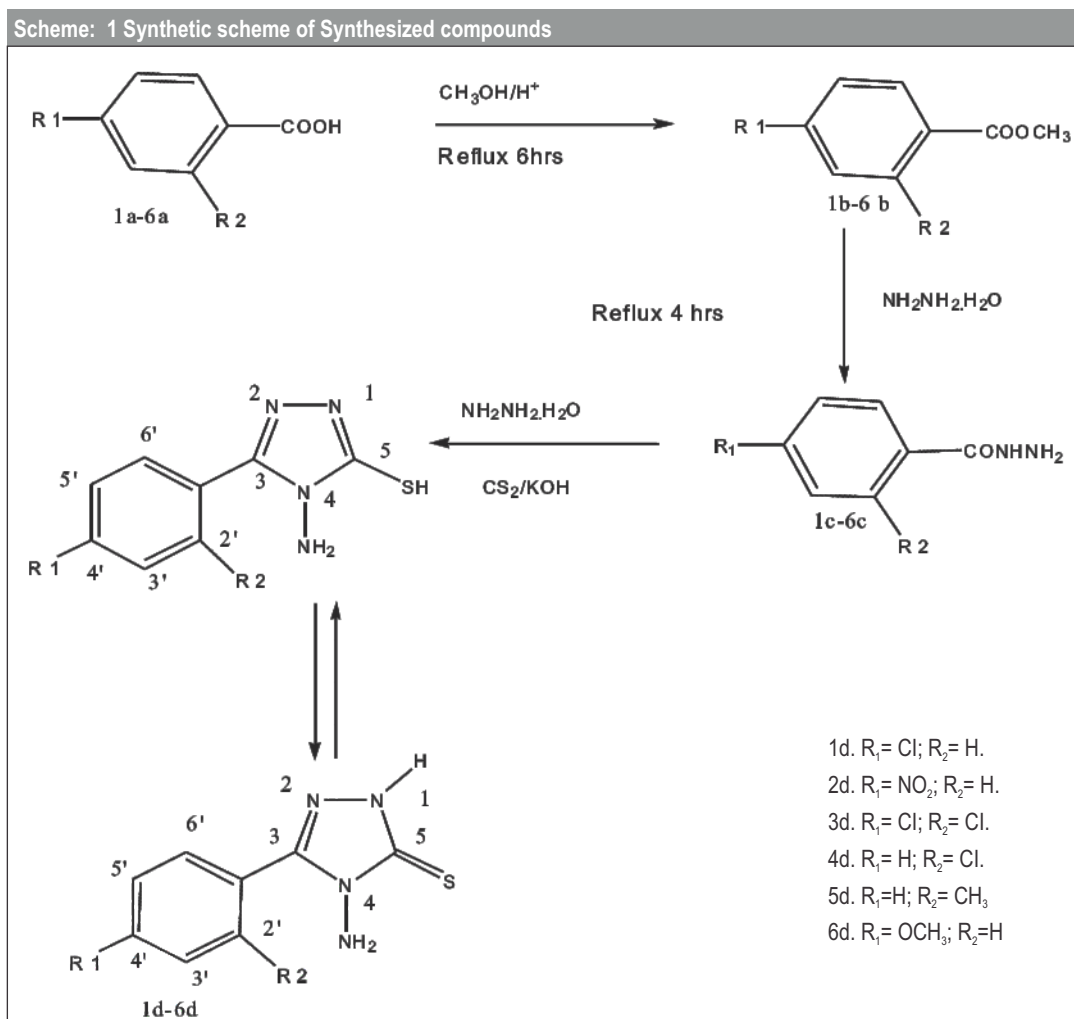
Group	Compounds	HB (g/dl)	MST	%ILS
I	Normal control	13.77 \pm 0.15	-	-
II	Induce control	4.57 \pm 0.12	16.15 \pm 0.11	-
III	1d	9.90 \pm 0.09*	21.51 \pm 0.05*	33.18
IV	2d	8.75 \pm 0.03*	19.23 \pm 0.13*	19.07
V	3d	10.20 \pm 0.11*	26.63 \pm 0.09*	64.89
VI	4d	11.30 \pm 0.10*	31.92 \pm 0.03*	97.64
VII	5d	5.60 \pm 0.05*	19.36 \pm 0.07*	21.17
VIII	6d	6.10 \pm 0.06*	18.21 \pm 0.03*	12.75
IX	5 FU	13.45 \pm 0.011*	41.70 \pm 0.15*	158.20

*P< 0.01, when compare all treated groups with the control in HB parameter and MST.

drug is the prolongation of the life span of animals. In case EAC-bearing mice, a regular rapid increase in ascites tumor volume was noted. Ascites fluid is the direct nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth indicated to meeting the nutritional requirement of tumor cells.

The enhanced survival time of the EAC bearing mice (Table 2) in all the drug treated groups indicates the anticancer efficacy of synthesized compounds. The compounds 4d and 3d were found to increase %ILS more significantly. It may be that decreased ascites fluid volume, viable cell count, or by decreasing the nutritional fluid volume and arresting the tumor growth increases the life span of EAC-bearing mice.

Myelosuppression and anemia (reduced hemoglobin) have been frequently observed in ascites carcinoma¹⁸. Anemia encountered in ascites carcinoma is mainly due to iron



deficiency, either by hemolytic or myelopathic conditions which finally lead to reduced RBC number¹⁷. In this study, we observed that elevated WBC count, reduced hemoglobin and RBC count in EAC control mice. All the triazole derivatives are tried to maintained normal hemoglobin level, normal values of RBC and WBC, thus prove that haematopoietic protecting activity of triazole derivatives.

Within the triazole series, it was noticed that the substituent at the position 3 with aromatic ring has great influence on the anticancer activity. These results may indicate the importance of chloro group at the of phenyl ring on the 1, 2, 4-triazoles. The compound **4d** substituted with 2-chlorophenyl at position 3 of the 1, 2, 4-triazoles found significant cytotoxicity to the EAC cells. It was showed in Table 1 that the percentage of ascites cell inhibition was 63.59%. In case of compounds **3d**, **1d**, **2d**, **5d** and **6d** substituted with 2, 4-dichlorophenyl, 4-chlorophenyl, 4-nitrophenyl, 2-methylphenyl and 4-methoxyphenyl at position 3 of the 1, 2, 4-triazole ring respectively a decrease in the cytotoxicity to the EAC cells

and the percentage of ascites cell inhibition respectively were (59.34%), (46.72%), (35.07%), (30.46%) and (21.23%).

CONCLUSION

By examining the anticancer activity of the compounds, we can deduce three main conclusions:

1, 2, 4-triazole derivatives possess antitumor activity consistent with those of some other derivatives drugs like oxadiazole derivatives & pyrimidine derivatives.

The presence of the 2-chlorophenyl at position 3 of the 1, 2, 4-triazoles ring, the compound **4d** is the most active cytotoxic among the tested compounds.

The reported results in this article may be a helpful guide for the researchers who are working in this area and 1, 2, 4-triazole with novel molecules for further modification as a potent anticancer agent.

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