IJPSR (2015), Vol. 6, Issue 8 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 28 January, 2015; received in revised form, 14 May, 2015; accepted, 15 July, 2015; published 01 August, 2015

EFFECT OF NOVEL BENZISOXAZOLE DERIVATIVES AGAINST EHRLICH ASCITES CARCINOMA CELLS IN SWISS ALBINO MICE: CYTOTOXIC AND HAEMATALOGICAL STUDIES

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Keywords:

Anticancer, Cytotoxic, EAC, MTT, WBC

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ABSTRACT: The present study is to investigate the cytotoxicity properties and haematological indices of synthesized molecules (S1-S4) in comparison to the standard drug 5 fluorouracil which reflects the anticancer potentials of the above molecules. Cytotoxicity studies were performed by in vitro MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl teterazolium bromide) assay. Haematological studies were done by drawing blood in the drug and synthesized molecules administered animals by retroorbital method. Haemoglobin, RBC, WBC, lymphocytes, Monocytes and Granulocytes counts were measured. All the above experiments were performed against Ehrlich ascites carcinoma (EAC) in Swiss albino mice. The percentage cytotoxicity and percentage viable cell counts revealed anticancer properties of the synthesized molecules. RBC count indicated protection of cell membrane from destruction and the WBC counts along with lymphocytes, Monocytes and granulocytes also show the immunoprotective characteristics of the novel molecules. Thus it has been concluded that the synthesized molecules (S1-S4) if supported by further molecular studies may have a promising role to play as anticancer agents.

INTRODUCTION: Cancer is one of the most devastating disease affecting humans, and the second most fatal disease after cardiac problems. Occurrence, development and decline of cancer cells are closely related. The current era beckons more efficient and safer therapeutic medications for amelioration of cancer. Synthetic derivatives have been known to play an important role in designing new drugs that prevent the onset of Cancer [1]. Thus there is an increasing interest in the biomedical and pharmacological investigation of synthetic molecules and their derivatives.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(8).3606-11

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(8).3606-11

Synthetic molecules with piperidine moiety have been shown to possess anticancer properties and one such known derivative is the benzisoxazole derivatives [2]. In this direction to investigate the cytotoxic and hematological properties the derivatives of 6-fluoro – 3 - (piperidin - 4-yl)benzo[d]isoxazole were synthesized [3], namely 4-(6-fluorobenzo [d] isoxazole - 3 - yl) – N - (3-methoxyphenyl)piperidine-1-carbothiamide (S1), N-(2 - chlorophenyl) - 4 - (6- fluorobenzo [d] isoxazole-3-yl) piperidine-1-carbothiamide (S2), 4-(6-fluorobenzo[d]isoxazole – 3 - yl) – N - (2-fluorophenyl)) piperidine-1-carbothiamide (S3), N-(4-chlorophenyl)-4-(6-fluorobenzo[d] isoxazole-3-yl) piperidine-1-carbothiamide (S4).

The Hematological indices like red blood cells (RBC), white blood cells (WBC) and hemoglobin were measured [4, 5]. Cytotoxic analysis was

performed by *in vitro* MTT assay [6]. MTT assay is known to be an important *in vitro* cytotoxic study to measure chemosensitvity in human cancer cell lines [7].

MATERIALS AND METHODS:

Animals:

Line bred female Swiss albino mice, 6-8 weeks old, weighing 20-25g were used for the present study. They were housed in standard environmental condition like, ambient temperature ($25^oC \pm 1^oC$), relative humidity (55±5%), and 24hr light dark cycle. Animals had free access to standard pellet diet and water *ad libitum*. All animal experiments were carried out in accordance with the guidelines of CPCSEA. The institute animal ethical committee has given the approval for the animals and the studies on them (approval No. HSK CP/IAEC/12013-14/121).

Acute Toxicity Study:

The dose selection was done out according to safe dose calculation as indicated in acute toxicity studies (OECD TG420) for the drug and samples S1 to S4. Acute toxicity studies gave similar results like that of anticancer drug. With this basis, we selected the dose 20 mg/kg for further studies.

Tumor cells:

Ehrlich Ascites Carcinoma (EAC) cells are mouse mammary carcinoma cells that grow ascites tumor in peritoneal cavity of mice. EAC cells were grown in the peritoneal cavity of six to eight weeks old Swiss albino mice by peritoneal transplantation of 0.5 ml of cell suspension contain 1×10^6 cells in sterile saline (0.9%NaCl).

Experimental design:

Male Swiss albino mice were divided in to 6 groups containing 12 animals in each group. The entire groups were injected with EAC cells ($1X10^6$cells/mouse) intraperitonealy.

Group I: Induced EAC cell ($1 X10^6$) with DMSO (0.9%)

Group II: Induced EAC cell ($1X10^6$) with 5fluorouracil (20mg/kg i.p)

Group III: Induced EAC cell ($1X10^6$) with S1 (20mg/kg p.o) with DMSO (0.9%)

Group IV: Induced EAC cell ($1X10^6$) with S2 (20mg/kg p.o) with DMSO (0.9%)

Group V: Induced EAC cell ($1X10^6$) with S3 (20mg/kg p.o) with DMSO (0.9%)

Group VI: Induced EAC cell ($1X10^6$) with S4 (20mg/kg p.o) with DMSO (0.9%)

In vitro anti cancer activity:

In vitro anticancer activity was conducted by MTT (3-(4, 5-dimethylthiazol-2-yl) - 2, 5- diphenyl teterazolium bromide) assay. Cells were grown in RPMI- 1640 medium at 37ºC and incubated at 5% CO_2 in a humified incubator for 6-7hrs. Cells were harvested, counted (3×10^4cells/ml), and transferred into a 24 well plate, and incubated for 24hrs. Prior to the addition of test compound. Serial dilutions of test samples were prepared by dissolving compounds in DMSO followed by dilution with RPMI-1640 medium to give final concentration at 20µg /ml. Stock solutions of samples were prepared. Sample at 10µl and cell lines at 90µl were incubated for 72hrs.

MTT solution at 5mg/ml was dissolved in 1ml of Phosphate Buffer Solution (PBS), and 10µl of it was added to each of the 24wells. The wells were wrapped with aluminum foil and incubated at 37ºC for 4hrs. The solution in each well containing media, unbound MTT and dead cells were removed by suction and 150µl of DMSO was added to each well. Then the plants were shaken and optical density was recorded using a microplate reader (spectrophotometer) at 595nm. Taking DMSO as blank, controls and samples were assayed and replicated for each concentration. After 24h incubation of the mononuclear cells with synthesized derivatives, the cytotoxicity on the cancer cell lines was evaluated using MTT assay. The cytotoxicity was obtained by comparing the absorbance between the samples and control. Cell viability (%) = Mean OD/Control OD x 100

Hematological studies:

In order to detect the influence of synthesized derivates (S1-S4) on hematological status of EAC bearing mice, a comparison was made among five groups (n = 6) of mice on the 15th day after inoculation. The groups were comprised of (I) Tumor bearing mice, (II) Tumor bearing mice

treated with 5- fluorouracil (20 mg/kg. i.p. . for 14 days). (III)S1 (20 mg/Kg/ day, p.o. for 14 days), (IV) S2 (20 mg/Kg/ day, p.o. for 14 days), (V) S3 (20 mg/Kg/ day, p.o. for 14 days) and (VI) Tumor bearing mice treated with S4 (20 mg/Kg/day, p.o. for 14 days).

Blood was drawn from each mouse by the retroorbital plexus method and the white blood cells (WBC), red blood cells (RBC) and hemoglobin and determined.

Statistical analysis:

All values were expressed as mean ± SEM. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by DMRT. P value<0.05 was considered as significant when compared to control.

RESULTS:

TABLE 1: EFFECT OF SYNTHETIC COMPOUNDS (S1-S4) AND STANDARD DRUG ON PERCENTAGE CYTOTOXICITY AND PERCENTAGE CELL VIABILITY IN EAC BEARING MICE

Parameter	Standard 5-fluorouracil	S1	S2	S3	S4
% Cell viability	14.17±0.48	34.33±1.05	19.17±0.60	23.33±0.88	28.17±0.65
% Cytotoxicity	85.83±0.48	65.67±1.05	80.83±0.60	76.67±0.88	71.83±0.65

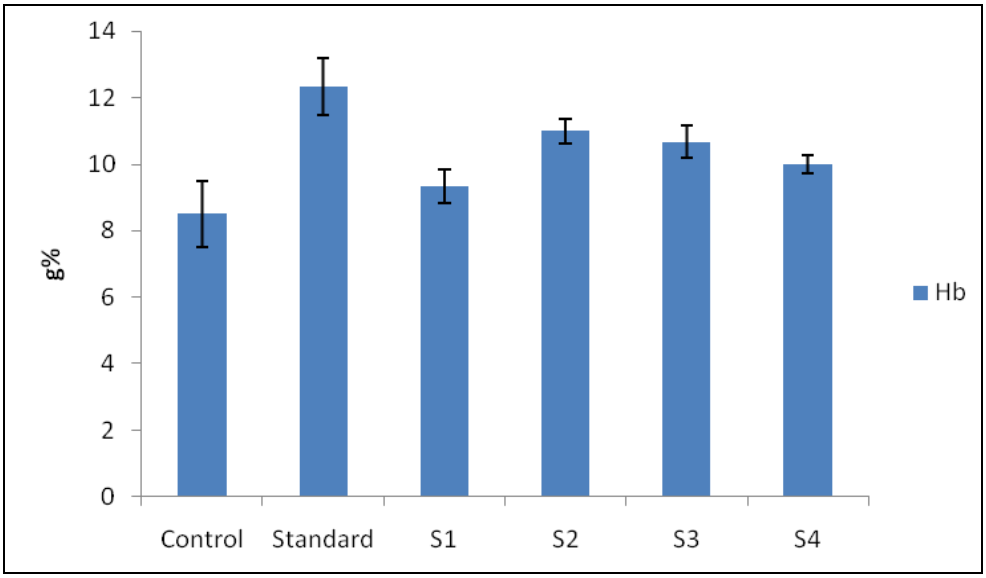

FIG. 1: EFFECT OF SYNTHETIC COMPOUNDS (S1-S4) AND STANDARD DRUG ON Hb CONCENTRATION

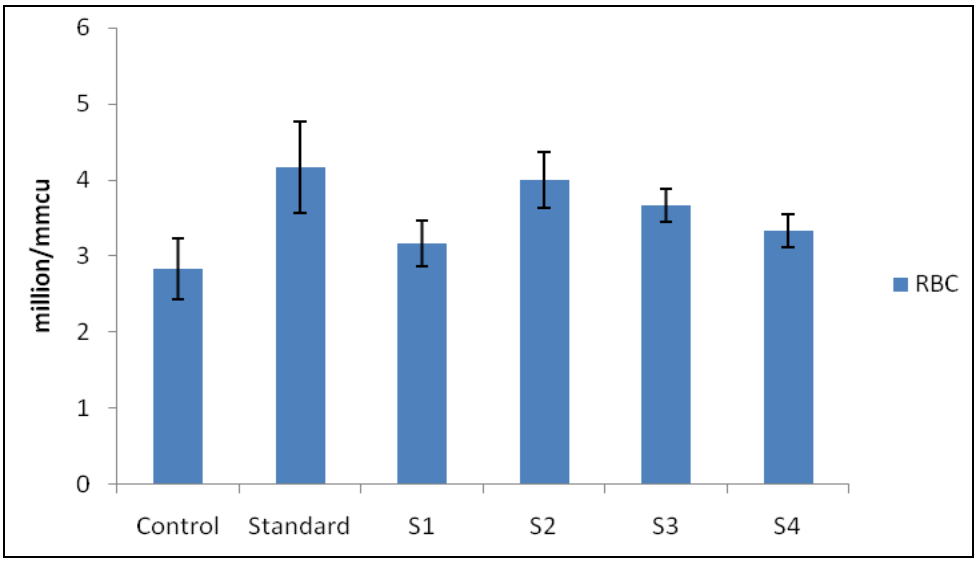

FIG. 2: EFFECT OF SYNTHETIC COMPOUNDS (S1-S4) AND STANDARD DRUG ON RBC CONCENTRATION

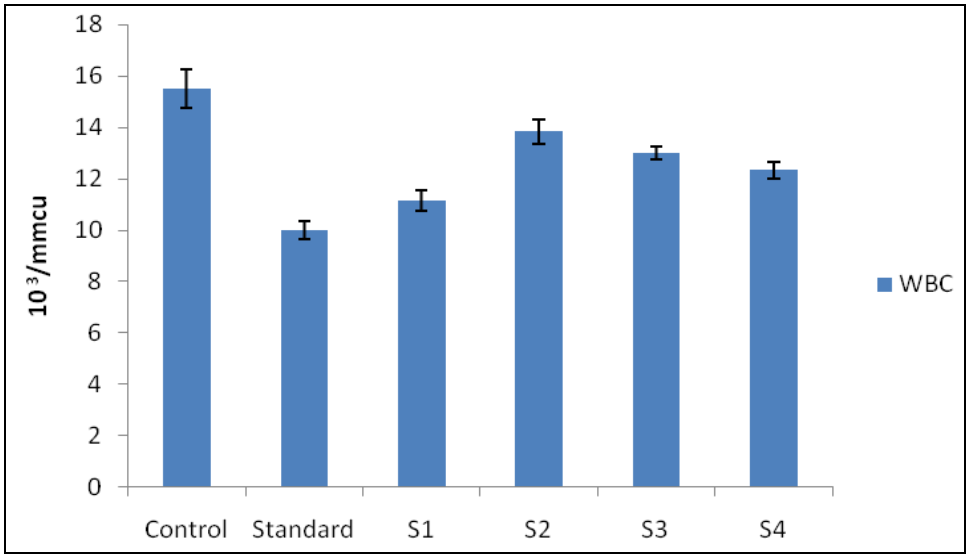

FIG.3: EFFECT OF SYNTHETIC COMPOUNDS (S1-S4) AND STANDARD DRUG ON WBC CONCENTRATION

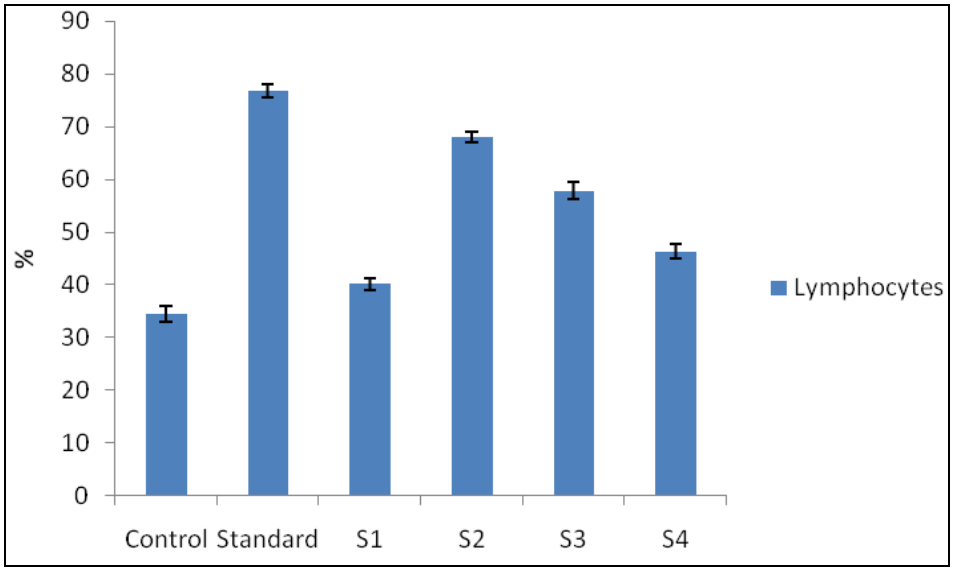

FIG.4: EFFECT OF SYNTHETIC COMPOUNDS (S1-S4) AND STANDARD DRUG ON LYMPHOCYTE CONCENTRATION

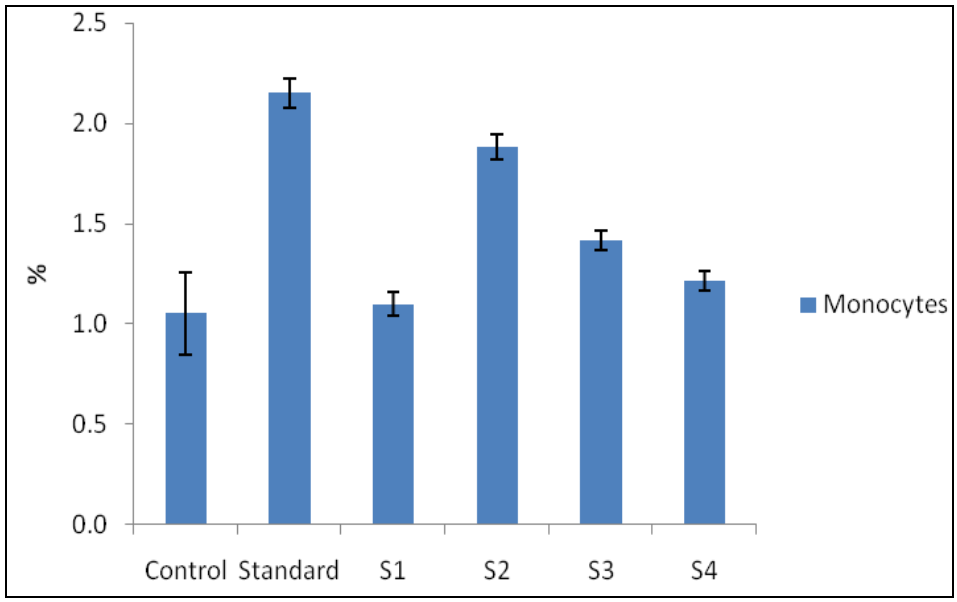

FIG.5: EFFECT OF SYNTHETIC COMPOUNDS (S1-S4) AND STANDARD DRUG ON MONOCYTES CONCENTRATION

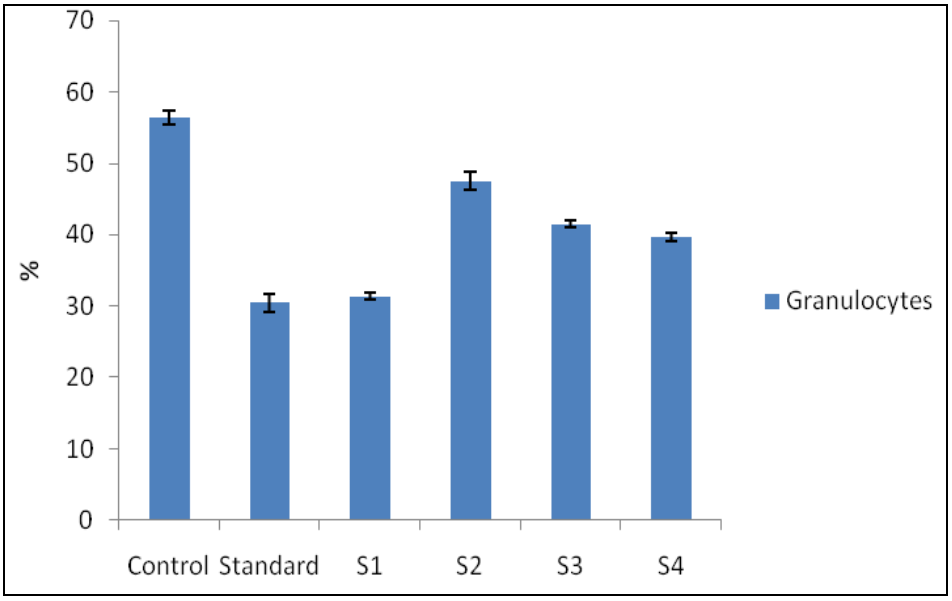

FIG.6: EFFECT OF SYNTHETIC COMPOUNDS (S1-S4) AND STANDARD DRUG ON GRANULOCYTES CONCENTRATION

DISCUSSION: The cytotoxicity studies of synthesized molecules (S1-S4) and standard drug 5-fluorouracil through MMT assay (**Table 1**) reveal significant findings with 5 fluorouracil (85.83±0.48) and S2 71.83±0.65 (80.83±0.60) showing activity percentage more than 80%. Followed by S3 (76.67±0.88), S4 (71.83±0.65) and S1 (65.67±1.05). Cell viability percentage also indicated complementary results to those of the above with standard drug (14.17±0.48) and S2 (19.17±0.60) showing viability less than 20%. However other molecules S1 (34.33±1.05), S3 (23.33±0.88) and S4 (28.17±0.65) also showed signs of promising cytoactivity with cell positive viability percentage. Thus suggesting variations in NAD(P) H-dependent cellular oxidoreductase enzyme levels [8]. This indicates the promising role of synthetic molecules particularly S2 and S3 as anticancer agents. Evaluation of hematological indices also showed significant results.

The values for haemoglobin (g%) (**Fig. 1**) content in S2 (11.00±0.37) and S3 (10.67±0.49) showed comparable values to that of 5 fluorouracil (12.33±0.84), however S1 (9.33±0.49) and S4 (10.00±0.26) also showed better results when compared to the normal (8.50±0.99). The RBC (million/mmcu) in **Fig. 2** also correlated with other results content with values for drug (4.17±0.60) and S2 (4.00±0.37) showing values more than 4 (million/mmcu), however values of S1 (3.17±0.31), S3 (3.67±0.21) and S4 (3.33±0.21) show higher values than the normal (2.83±0.40). Thus Haemoglobin (g%) content and RBC count values indicate cytoprotective effects of the synthesized molecules (S1-S4), which otherwise in the normal EAC carrying mice showed destruction of RBC, thus decreased count [9]. The WBC values are important criteria to determine the anticancer properties of the administered molecules [10]. WBC count (10^3/mmcu) of drug (10.00±0.37) according to **Fig. 3** was similar to synthesized molecules S2 (13.83±0.48) and S3 (13.00±0.26). Other synthesized molecules S1 (11.17±0.40) and S4 (12.33±0.33) also showed improved results than normal (15.50±0.76). The lymphocyte percentage (**Fig. 4**) also indicated elevated results for standard drugs (76.83±1.28), S2 (68.00±0.93) and S3 (57.83±1.60) when compared to the EAC control (34.50±1.61)[11].

In **Fig. 5** Monocytes percentage [12] for S2 (1.88±0.06) was very similar to that of standard drug (2.15±0.08) remarkably higher than the normal (1.05±0.21). However as seen in **Fig.6** granulocytes percentage [13] of S1 (31.33±0.49) was comparable to that of standard drug (30.50±1.26). All the above results for haematological studies reflect the immune protective properties of synthesized molecules (S1-S4), particularly S2 in comparison to the standard drug 5 fluorouracil.

CONCLUSION: Based on the above results we can conclude that synthesized molecules (S1-S4),

particularly S2 and S3 have shown comparable results to that of standard drug 5 fluorouracil thus exhibiting cytoprotective properties. Haematological studies also indicate anticancer properties when investigated against EAC in Swiss albino mice. However higher experimental models are required to further investigate their anticancer potential which may lead to design new anticancer drugs.

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How to cite this article:
Sharath Chandra SP and Mahadimane PV: Effect of Novel Benzisoxazole Derivatives against Ehrlich Ascites Carcinoma Cells in Swiss Albino Mice: Cytotoxic and Haematalogical Studies. Int J Pharm Sci Res 2015; 6(8): 3606-11.doi: 10.13040/IJPSR.0975-8232.6(8).3606-11.