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Biochemical Studies of (5-P-chlorophenyl -2-benzo 5, 6-coumarin-3-yelthylidene aminothiazole) as Antitumor Agent

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Heterocyclic compounds have a large spectrum of biological activities including antitumor activity. The present study describes the cytotoxic effect of newly synthesized thiazole derivative (TD2) that can prove effective antitumor activity on both in vivo and *in vitro* studies.

Objective: the essential objective of this research is to prove the cytotoxic effect of newly synthesized thiazole derivative (TD2) up on EAC bearing mice and many kinds of human cell lines.

Materials and Methods: Antitumor activity of TD2 was examined on EAC in Swiss albino Mice at dose of 2.5 mg/kg. TD2 was injected for 10 following days after transplantation of tumor. After one day of last dose and 18 hours of fasting, 7 Mice were sacrificed and the remaining was kept to evaluate ILS %.

Antitumor activity of TD2 was assessed by inspecting tumor volume, tumor weight, viable cell count and nonviable cell count, hematological, biochemical and antioxidant parameters of mice.

Results: TD2 demonstrated an inhibitory effect on both cancer cell lines *in vitro* and Ehrlich ascites cells *in vivo*.TD2 increased in life span of Ehrlich–bearing mice compared to control. Cell cycle and

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flow cytometric analysis revealed that TD2 directed Ehrlich cells toward apoptosis by increasing of P_{53} expression.
Conclusion: It was concluded that TD2 have a potent antitumor activity against Ehrlich ascites carcinoma in mice beside a cytotoxic effect on MCF-7, PC3, HepG2 and HCT-116.

Keywords: 2,5- disubstituted thiazoles; cytotoxicity; antioxidant; antitumor; Ehrlich ascites Carcinoma; flow cytometry; in vivo; in vitro; TD2; P53.

1. INTRODUCTION

Cancer is an abnormal proliferation of a group of cells which can metastases in other body organs. Carcinogenesis is a process that leads to genetic mutations which is induced by physical agents (irradiations), chemical agents (food, smoking and drugs), some viruses (hepatitis B virus and HIV), some bacteria (H. pylori), and other intrinsic factors as age, sex and growth factors [1].

Thiazole derivatives are known to possess several biological activities including anticancer activity [2].There are a variety of mechanisms for the antitumor action of thiazole derivatives, acting on cancer bio targets, such as tumor necrosis factor TNF-α [3], inosine monophosphate dehydrogenase (IMPDH) [4] and apoptosis inducers [5].

Many cancers have the tumor suppressor p53 inactivated by mutation, making reactivation of mutant p53 with small molecules a promising strategy for the development of novel anticancer therapeutics. The oncogenic p53 mutation Y220C, which accounts for approximately 100,000 cancer cases per year, creates an extended surface crevice in the DNA-binding domain, which destabilizes p53 and causes denaturation and aggregation. Aminobenzothiazole derivative, binds tightly to the Y220C pocket and stabilizes p53-Y220C *in vitro*.

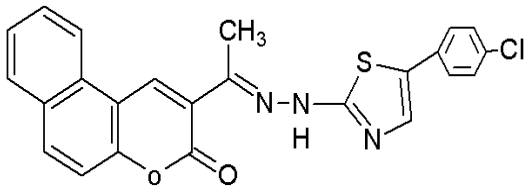
2. MATERIALS AND METHODS

2.1 Chemicals

The following chemicals was used in the synthesis of thiazole derivative: 2-Hydroxynaphthaldehyde, 2,3-dihydroxybenzaldehyde, thiosemi carbazide, p-chloroacetophenone, bromine, ethyl acetoacetate, piperidine,glacial acetic acid, ethanol (100%), DMSO, propidium iodide (PI)stock solution were purchased from Acros Organics (Geel, Belgium). Phosphate buffer saline was purchased from Biowest company – Egypt. ribonuclease I and 5-fluorouracil (5-FU) were purchased from Sigma-Aldrich (R&D system, USA). Biochemical kits were purchased from Spinreact Company. Antioxidant kits were purchased from Biodiagonistic Company – Egypt.

2.2 Synthesis of the Compounds

One Compound of thiazole derivative was prepared in the faculty of science, Port Said University and was taken abbreviation (TD2) which is:-



5-P-chlorophenyl-2-benzo 5, 6-coumarin-3-yelthylidene amino thiazole

2.3 Synthesis of Thiosemicarbazone Derivative (III)

In a 150 ml measuring flask acetylbenzocoumarine (3.01 mol) (III) and glacial acetic acid (25 ml) were added to the thiosemicabazide (1.01 mol) (I).the reaction mixture was heated under reflux for 2 hrs. after cooling, the contents were quenched with cold water .solid products were collected after filtration, washing with water, dried and recrystallized with suitable solvent to give thiosemicarbazone derivatives (IV&V) (Scheme 1) [6].

2.4 Synthesis of Thiazole Derivatives (V)

In a 250 ml measuring flask, p- chlorophenacyl bromide (6.01 mol)(IV), fused sodium acetate (0.03 mol) and ethanol (70 ml) were added to the thiosemicarbazone derivative (III) (0.01 mol). The

reaction mixture was heated under reflux for 4 hrs. After cooling, the contents were poured in to ice–water. The resulting product was filtered off , washed with water, dried and purified by recrystallization from ethanol to give and 5-p-chlorophenyl-2-benzo 5,6-coumarin-3-yelthylidene aminothiazole (V) (TD2) (Scheme 2) [6].

2.5 Synthesis of Potassium salts of Thiazole Derivatives (VI)

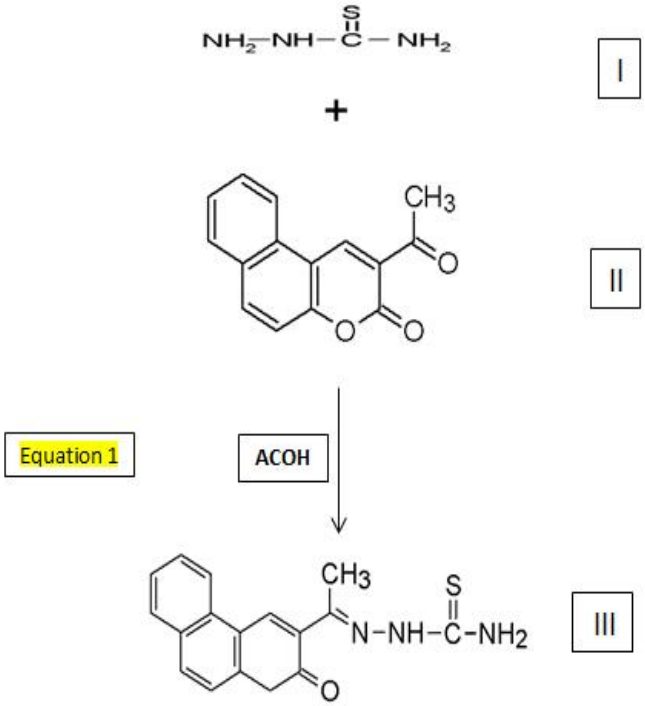
0.01 mole of thiazole derivative (V) were dissolved in 30 ml aqueous potassium hydroxide (0.03 mole) in beaker. The solution was left in open beaker for 2-3 days, till the solvent was evaporated, the solid residue is the potassium salt for the thiazole derivative (Scheme 2).

2.6 *In vitro* Study of Cytotoxicity

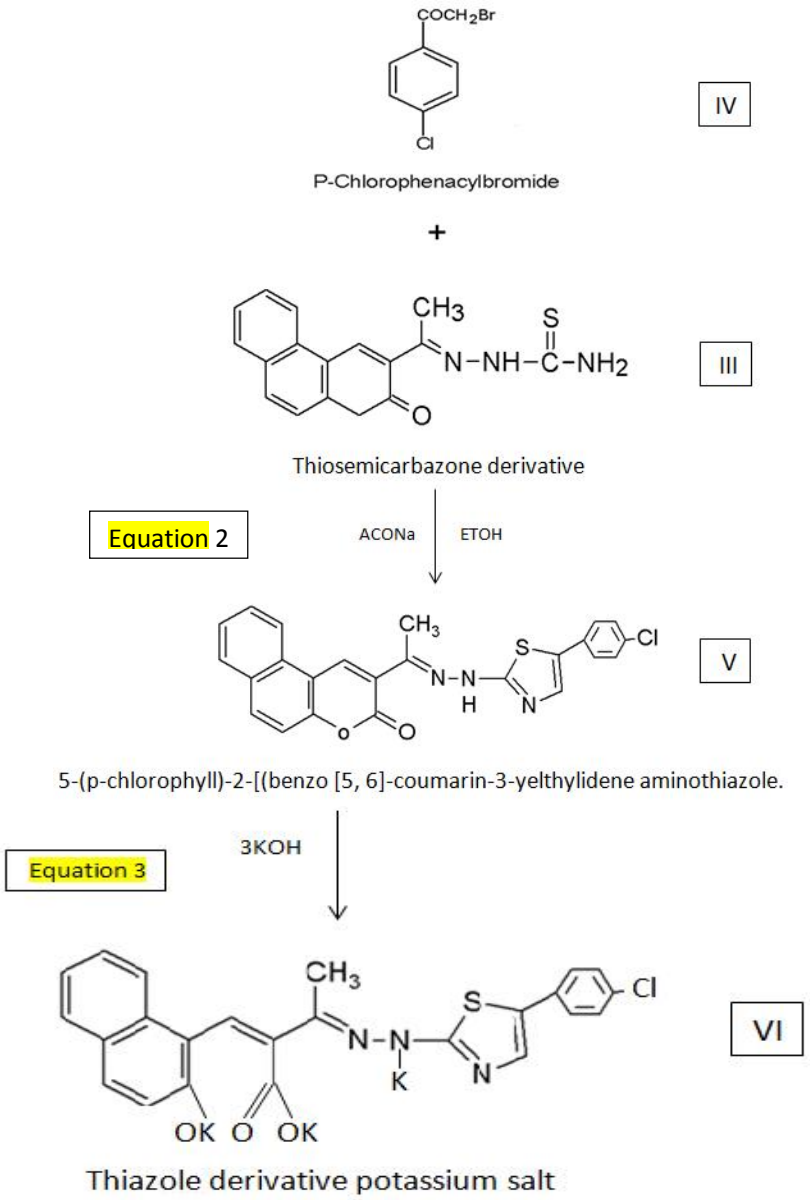
The cytotoxic effect of TD2 on HCT-116 (colon cancer), MCF-7 (breast cancer), HepG2 (liver cancer) and PC-3 (prostate cancer) were evaluated by determination of IC_{50} value that indicates to the concentration of compound causing 50% death in the population of cancer cells.

The *in vitro* anticancer screening was performed in the pharmacology unit at the National Cancer Institute, Cairo University according to the following skehan protocol.

Cells were plated in 96-multiwell plate (104 cells/ well) for 24 hrs before treatment with the compounds to allow attachment of cell to the wall of the plate. Test compounds were dissolved in dimethylsulfoxide (DMSO) and diluted with saline to the appropriate volume. Different concentrations of the compounds under test (10, 25, 50 and 100 µM) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 hrs at 37 0C and in atmosphere of 5% CO2.After 48 hrs, cells were fixed, washed and stained for 30 min. with 0.4% (w/ v) Rhodamine-B stain dissolved in 1% acetic acid. Unbounded dye was removed by washing four times with 1% acetic acid, and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an enzyme linked immunosorbent assay (ELISA) reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC50) was calculated.



Scheme 1. Synthesis of thiosemicarbazone derivative (III)



Scheme 2. Synthesis of thiazole derivative (V) and its potassium salt (VI)

2.7 *In vivo* Study

2.7.1 Determination of median lethal dose (LD50)

LD50 of TD2 was determined according to Chinedu method Eight mice (four mice for each compound) received the test compounds at doses 10,100,300 and 600 mg/ kg / b.w. In the first day, the animals were observed for one hour post administration and then 10 minutes every 2 hours interval for 24 hours. LD50 was calculated by the following formula:

$$LD_{50} = \frac{[M_0 + M_1]}{2}$$

Where,

M0 = Highest dose of test substance that gave no mortality,
M1 = Lowest dose of test substance that gave mortality.

2.7.2 Animals

150 healthy female Swiss albino mice of about 10 weeks old weighing 25-30 g were housed in polypropylene cages under standard conditions (12/12 h light and dark cycle, temperature 25±4°C, water *ad libitum*. The mice were maintained according to guidelines approved by Animal Ethical Committee at faculty of science, Zagazig University.

2.7.3 Tumor model

Ehrlich ascites carcinoma (EAC) bearing mice were firstly obtained from National Cancer Institute (NCI), Cairo. EAC were injected intraperitoneally once at the first day of experiment and were allowed to grow in the peritoneal cavity of mouse for ten days.

2.7.4 Experimental design

Mice were randomly divided into four groups (15 mice per group) as follows: -

Group I (Negative control): mice were injected intrapretonially with saline only.

Group II (Positive control): mice were injected intrapretonially with EAC (4× 10^6 cells/ml).

Group III (drug control): mice were injected with EAC (4×10^6 cells) once in the first day, IP + 5 florouracil was injected (20 mg/kg, day after day for 10 days), IP.

Group IV: mice were injected with EAC (4×10^6 cells) once in the first day, IP + TD2 (5 mg/kg, day after day for 10 days).

In the eleventh day, mice were anesthetized and blood was collected in EDTA-tube by retro orbital plexus using capillary tube, and then stored at -20°C until using. In addition, blood without anticoagulant was collected in plain tube and left for 10 minutes in water bath at 37°C, then centrifuged for serum separation. After sacrificing of mice, Ehrlich ascetic fluid was collected for determining the viability of cells, the expression of p^{53} and cell cycle analysis.

Finally, liver and kidney samples were collected in saline containing jars for estimation of antioxidant status and small pieces were fixed in 10% formalin for histological study. Seven mice per each group were left till death to determine the mean survival time (MST) and increase in life span percentage (%ILS) (Fig. 1).

2.7.5 Evaluation of tumor weight

The weight of mice was estimated before and after withdrawal of EAC fluid and the deference is the increase in weight and expressed in grams.

2.7.6 Evaluation of Tumor volume

The volume of the EAC was detected by measuring tube in milliliters (ml) [7].

2.7.7 Determination of cell viability

100 µl of Ehrlich ascites cells were stained with trypan blue for 15 minutes and the number of viable cells were counted using homocytometer according to the following formula;

Number of cells / ml = average × 25 ×10^4× dilution factor

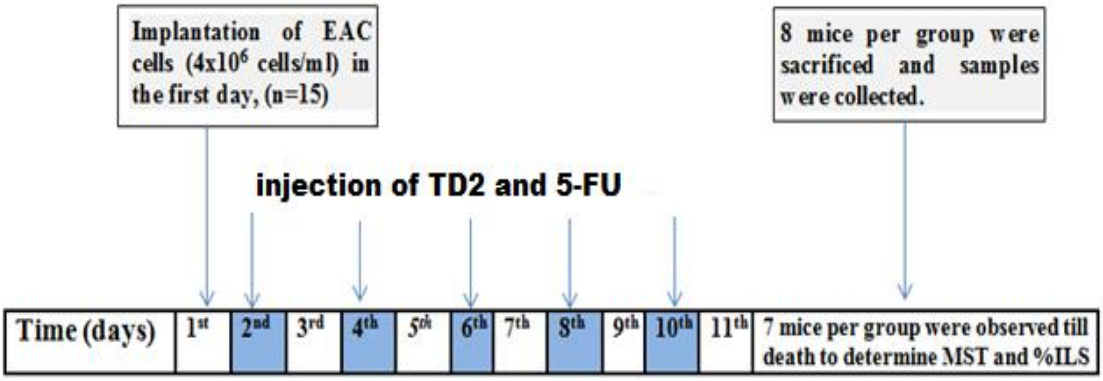


Fig. 1. Experimental design

2.7.8 Determination of percentage increase in life span (%ILS)

Mean survival time (MST) and %ILS for each group were calculated according to the following formula, [7].

MST= (no. of days to 1st death + no. of days to last death) / 2

%ILS= [(MST of drug group / MST of control group)-1] × 100.

2.7.9 Evaluation of hematological parameters

Complete blood count was performed using automated blood analyzer (Mindray BC2800, UK).

2.7.10 Evaluation of biochemical parameters

Alanine transaminase (ALT), aspartate transaminase (AST) activities and the concentration of albumin, total bilirubin, urea, creatinine, cholesterol and triacylglycerol in serum were determined by colorimetric methods according to manufacturer's instructions (Spinreact, Spain) using semi-automated biochemistry analyzer (Robonik, India).

2.7.11 Evaluation of antioxidant parameters

The activity of Super oxide dismutase (SOD), Catalase (CAT) and reduced glutathione (GSH) and malondialdehyde (MDA) levels were determined by commercial kits (Biodiagonistics, Egypt) according to manufacturer's instructions.

2.8 Histological Study

Liver and kidney tissues were fixed in 10% formaline, then sectioned in paraffin wax and examined under light microscope after staining with Hematoxylin and eosin.

2.9 Statistical Analysis

Statistical analysis was carried out using SPSS program version 14. Data were expressed as mean ± SE. Student (t) test and One-way ANOVA test were performed to detect the significance variation between test groups and control.

3. RESULTS

3.1 Chemistry

1-[(Benzocoumarin-3-ylethylidine) amino]-1,3-thiourea (compound V) as a pale yellow crystals, yield 79% , M.P.251°C, IR (KBr) :3320, 3158 (NH2), 3225 (NH),1732 (C=O). 1631 (C=N),1605, 1588 (C=C) 1476 (C=S), 1098 (C-O) cm-1.1H–NMR (DMSO-D6):S 2.2 H (S,3H,CH3), 6.63(N,2H, NH2),7.12-8.01(M,6H,AR-H),8.51(S,1H,CH of pyrane), 10.40 (S,1H,NH) ppm. Anal. Calcd. For $C_{16}H_{13}N_3O_2S$ (311,C,61.74:H,4.18:N,13-50 found : C, 61.47 : H,4.03:N,13.25.

5-P-Chlorophenyl-3-benzocoumarin-3-ylethylidineamino-1,3-thiazole (TD2) (V) obtained as yellow crystals, yield 72%, M.P.205°C, IR (KBr) :3222 (NH) ,1733 (CO of pyranone ring),1623(C=N),1605,1595 (C=C),1167,1095 (C-O) cm-1,1H–NMR(DMSO-D6): s 2.24 (S,3H,CH3) , 6.89-8.1(M,10,Ar-H and H – thiazole), 8.53(S,1H,H of pyranone ring),11.97 (S,1H,NH) ppm, MS:M/Z(%) = 447(M++2,17-30), 445 (M+,52.6), 444 (M+-1,16-20), 236 (11.20),235 (17.20), 221(17.20), 220 (6.30), 212(33.20), 210(100),170 (7.5), 168 (22.50) ,137(11.20),136(33.20),113(6.20),111(18.20). Anal calcd. for $C_{14}H_{16}N_3CLO_2S$ (445): C,64.72: H,3.59:N,9.44. found: C,64.48:H3.36:N,9.22.

3.2 Results of *In vitro* Cytotoxicity

Fig. 2 (a,b,c,d,) demonstrated the cytotoxic effect of TD2 on different human cancer cell lines and IC50 values were determined as showing in Table 1.

Table 1. Demonstration of IC_{50} (µg/ml) of TD2 on human cancer cell lines

Cancer cell line	IC_{50} (µg/ml) ± SD
HCT116	20 ±2.1
MCF7	21.4 ± 1.5
HEPG2	19.6 ± 1.45
PC3	Non determinant

3.3 LD_{50} of TD2

2.7.1 Determination of median lethal dose (LD50)

LD50 of TD2 was determined according to Chinedu. Eight mice (four mice for each compound) received the test compounds at doses 10,100,300 and 600 mg/ kg / b.w. In the first day, the animals were observed for one hour post administration and then 10 minutes every 2

hours interval for 24 hours. LD50 was calculated by the following formula:

$$LD_{50} = \frac{[M_0 + M_1]}{2}$$

Where,

M0 = Highest dose of test substance that gave no mortality,
M1 = Lowest dose of test substance that gave mortality.

3.4 Dose Response Curve

According to dose response curve as shown in Fig. 3 effective dose of TD2 against EAC was recorded at 2.5 mg /kg.

3.5 The Effect of Test Compounds (TD2) on Tumor Volume, Viability of EAC Cells and Life Span Prolongation

The number of viable EAC cells decreased by 90.75% ($P < 0.001$) after administration of TD2, when compared to EAC control group. Moreover, the percent increase in life span reached 40% ($P<0.001$) in the group which received TD2 compared to EAC control group Table 2.

3.6 The Effect of TD2 on Hematology

As shown in Table 3, RBCs count was increased in TD2 groups by 51% compared to EAC control group, while hemoglobin concentration was increased in TD2 group by 15% as compared to EAC control group. In addition, administration of TD2 raised WBCs count by 1000% compared to EAC control group (Table 3).

3.7 The Effect of TD2 on Biochemical Parameters

Results of the present study demonstrated that the liver activities of AST (aspartate aminotransferase) and ALT (Alanine aminotransferase) were increased in EAC – bearing mice when compared to normal group. Administration of TD2 decreased ALT concentration by 16% compared to control group. So, administration of TD2 was slightly decreased AST concentration by 0.4%, compared to control group (Table 4).

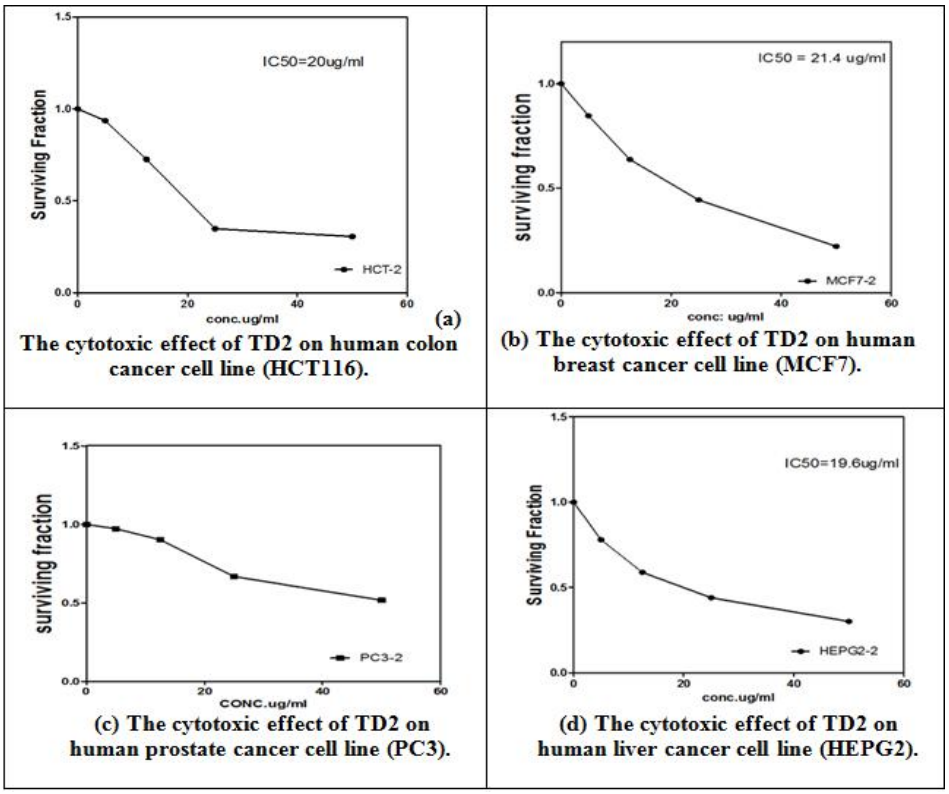


Fig. 2. The cytotoxic effect of TD2 on human cancer cell lines (HCT116, MCF7, HepG2 and PC3)

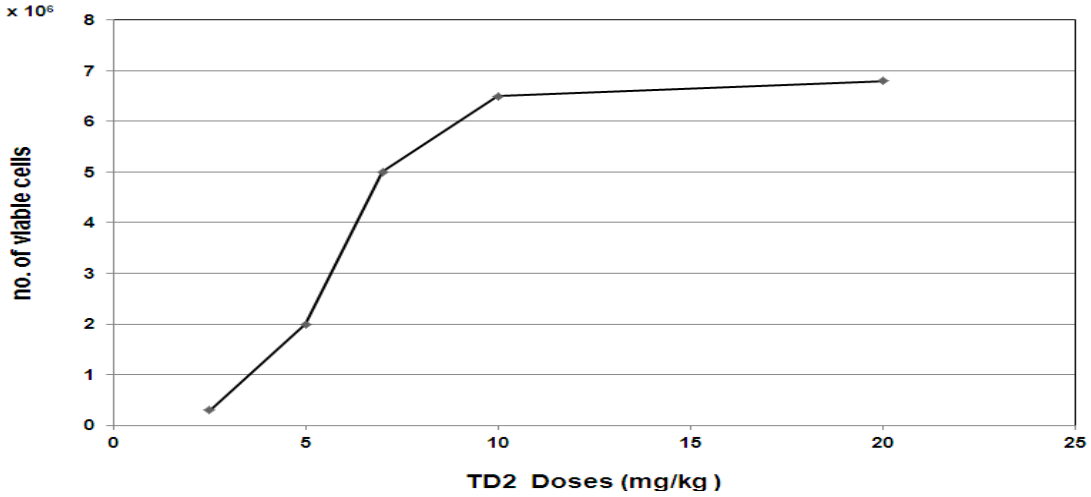


Fig. 3. Dose response curve for and TD2

3.8 The Effect of TD2 on the Oxidative Status in Liver

Catalase, GSH and SOD levels are increased in EAC control group comparing with normal control group.

Normalization of these changes was determined after treatment with TD2 at dose 2 .5 mg/kg, While MDA (malondialdehyde) was increased in EAC control group and lowered in drug group demonstrating the potential of TD2 as antioxidant agents as shown in Table 5.

3.9 Results of Histology

It was found that the histological architecture of liver and kidney tissues in mice which received EAC only, interrupted severely while mice which treated with TD2 after EAC inoculation demonstrated an improvement in the histological characteristics of liver and kidney tissues as shown in Figs. [4-11].

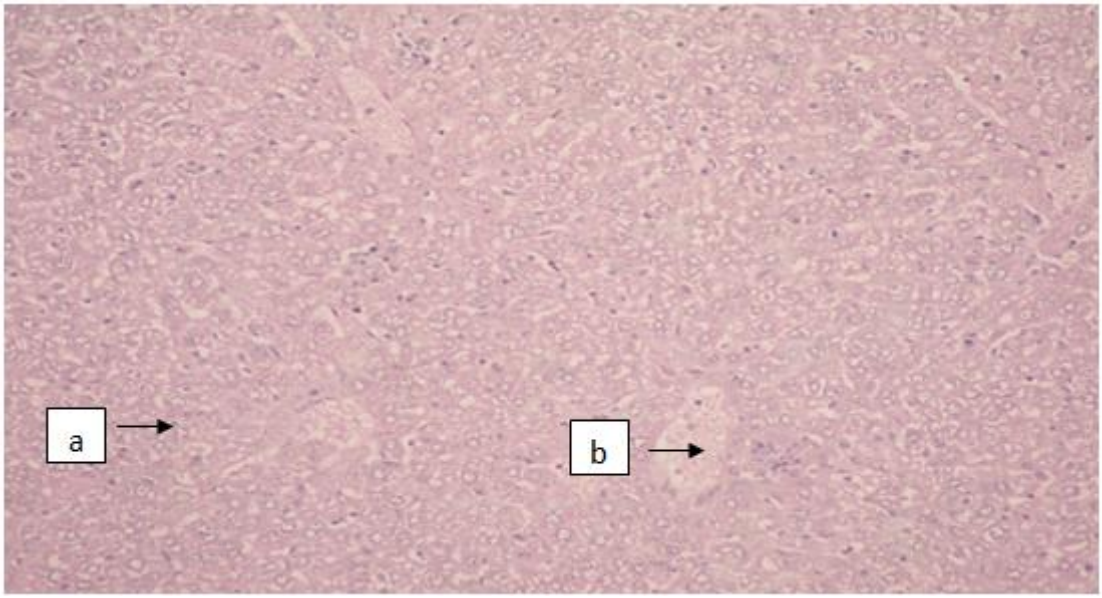


Fig. 4. Photomicrographs of liver section of mice injected with normal saline only showed: - (a) Euchromatic nuclei and (b) Central vein radiating coats of hepatocytes (40x)

Table 2. Effect of TD2 on tumor volume (ml), cell viability, mean survival time, percent increase in life span

Groups / indicators	EAC Control M±SE	EAC+ TD2 (2.5 mg/kg) M±SE	EAC+5-FU (20 mg/kg) M±SE
Tumor volume (ml)	1.9 ± 0.48	0.7*** ±0.15	0.3*** ±0.48
Viable cell (x10^6)	39.5 ± 0.81	4.05*** ±0.11	11*** ±0.23
Ascites inhibition rate (%)	-	90.75	72.5
MST(days)	15 ± 0.45	21***±0.12	24***±0.29
ILS%	0	40.00	66.66

*Values are represented as mean ± SE (n=7). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 as compared to EAC control group. MST=Mean survival time, %ILS=Percentage increase life span, EAC=Ehrlich ascites carcinoma TD2 =Thiazole Derivative 2*

Table 3. The effect of test compounds (TD2) on hematological parameters

Groups / Indicators	Saline M±SE	EAC M±SE	EAC+TD2 M±SE	EAC+5-FU M±SE
WBCs	11.1±0.12	2.87±0.03	33.86*** ±2.4	5.4*** ±0.33
RBCs	6.84±0.75	4.7±0.89	7.1*±0.12	6.4*** ±1.14
HGB	10.7±0.25	8.3±0.44	9.5** ±0.67	10.5* ±1.63
HCT	35.0±0.11	19.9±0.63	32.5** ±0.13	32.8*** ±1.1
MCV	51.2±0.12	42.2±0.37	45.8*** ±0.77	51.2±0.55
MCH	15.6±0.73	17.6±0.69	13.4±0.07	16.4*** ±0.12
MCHC	30.4±0.23	41.7±0.87	29.2*** ±2.45	32** ±0.17

*Values are represented as mean ±SE (n=7), Where * = Significant difference between test groups and EAC group,*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001*

Table 4. The effect of TD2 on serum biochemical parameters

Group	ALT (U/L) M±SE	AST (U/L) M±SE	T.Bil (mg/dl) M±SE	Albumin (g/l) M±SE	Urea (mg/dl) M±SE	Creatinine (mg/dl) M±SE	Cholestrol (mg/dl) M±SE	Triglyceride (mg/dl) M±SE
Saline	39.55 ±1.13	218.69 ±10.49	0.45 ±0.04	3.098 ±.238	24.65 ±1.2	0.86 ±0.038	101.66 ±0.74	275.26 ±10.92
EAC	50.35 ±1.81	399 ±49.82	0.46 ±.037	2.33 ±0.35	36.95 ±2.49	0.56 ±0.035	83.59 ±2.8	114.90 ±1.18
EAC+TD2	42.40 ±3	397.4 ±49.82	0.47 ±.026	2.63 ±.027	59.77* ±2.53	1.02* ±0.07	66.45* ±2.6	164.4* ±23.02
EAC+5-FU	43.48** ±0.48	186.2*** ±1.06	0.45*** ±0.95	3.02 ±0.5	13.2* ± 0.12	0.42*** ±1.94	47.55 ±1.24	133.5*** ±0.66

*Values are represented as mean ±SE (n=7), Where * = Significant difference between test groups and EAC group,*P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001.where : ALT: Alanine aminotransferase, AST: of aspartate aminotransferase, T.Bil: total bilirubin*

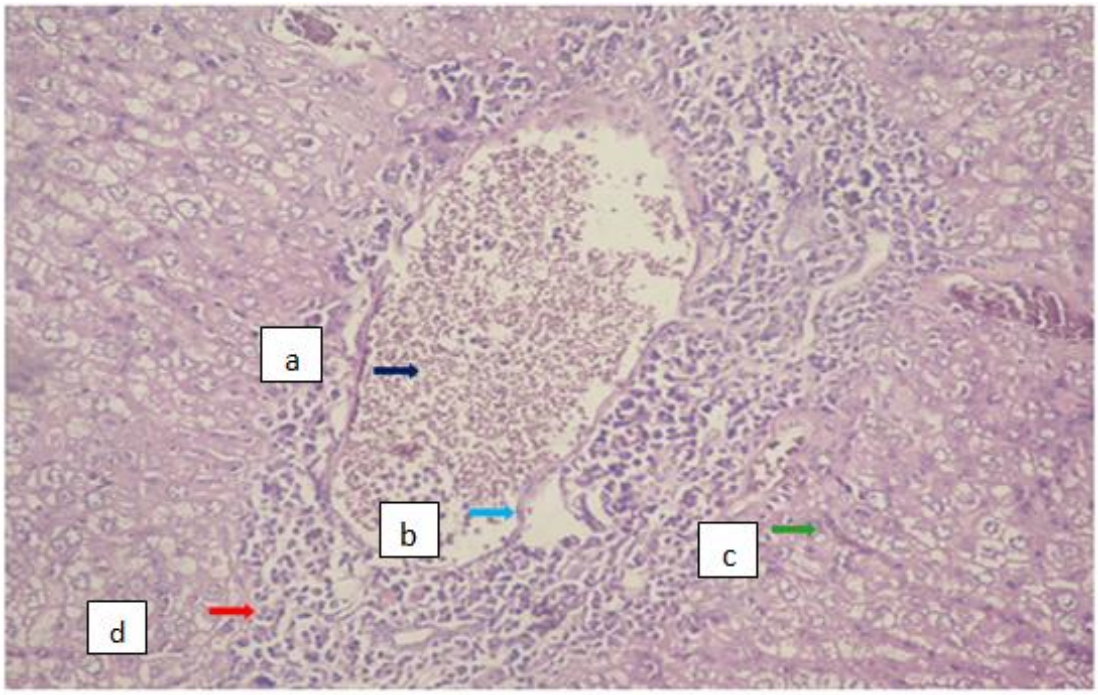


Fig. 5. Photomicrographs of liver section of mice injected with EAC only showed: - (a) congested Central vein, (b) exfoliated Epithelial cells, (c) congested Sinusoids and(d) Mitotic figures (40x)

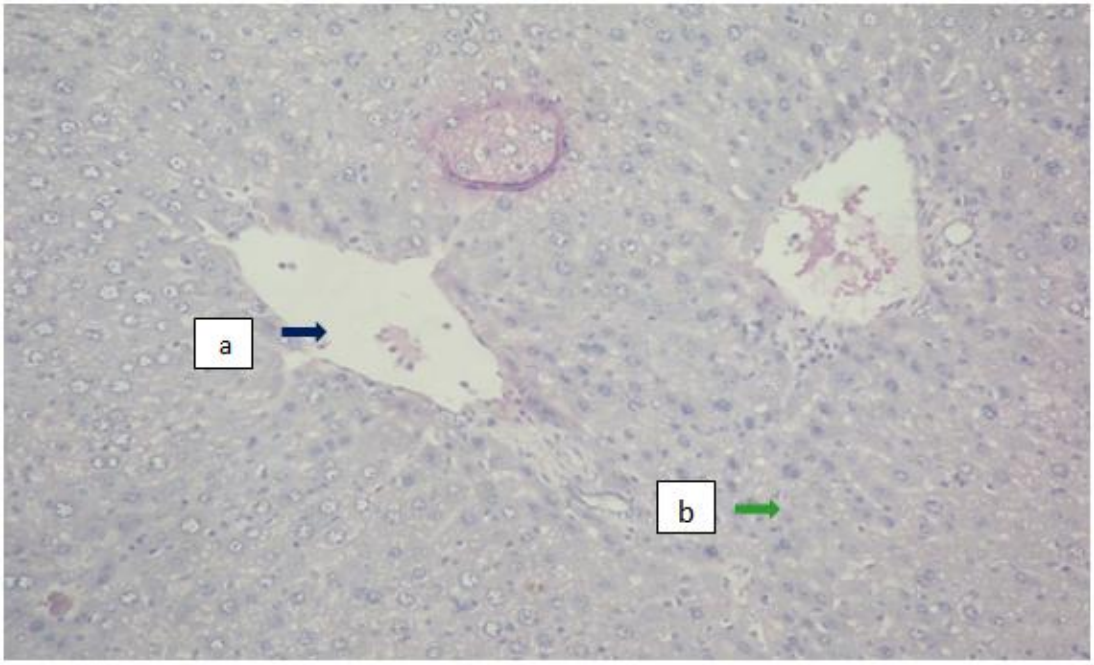


Fig. 6. Photomicrographs of liver section of mice injected with EAC + TD2 showed: - (a) Central vein normal epithelial lining with Mild congestion and (b) normal arrangement of Hepatocytes with active nuclear (40x)

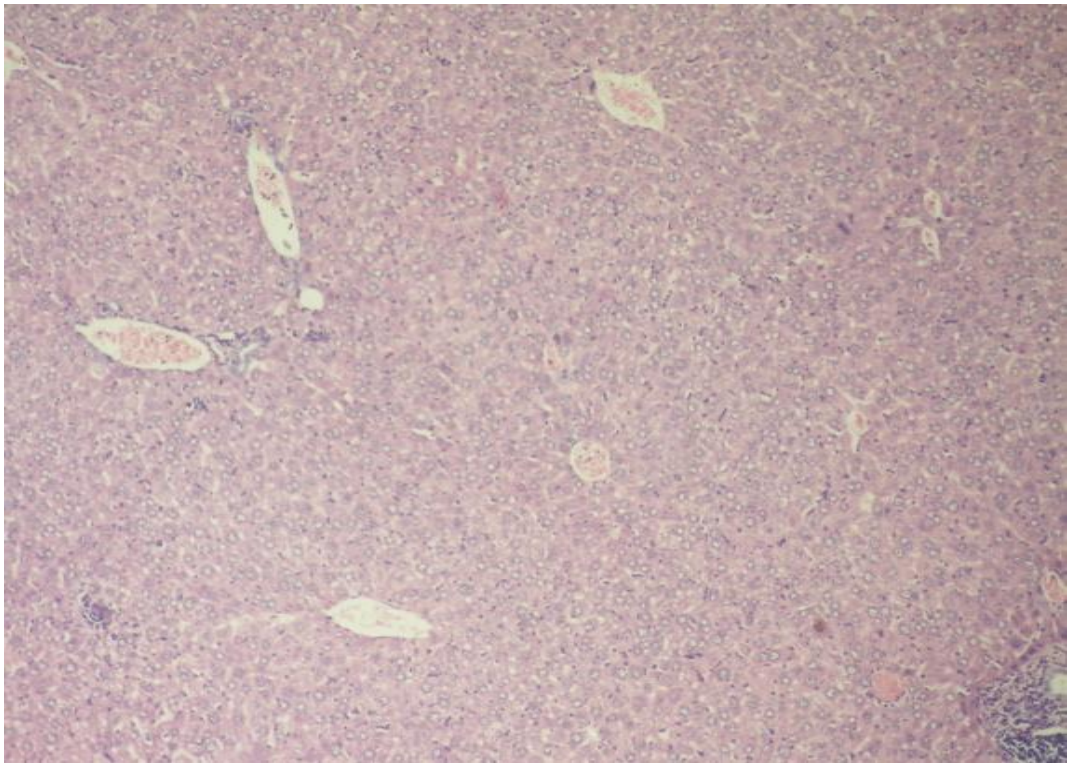


Fig. 7. Photomicrographs of liver section of mice injected with (EAC+5FU) showed normal liver cells. (40x)

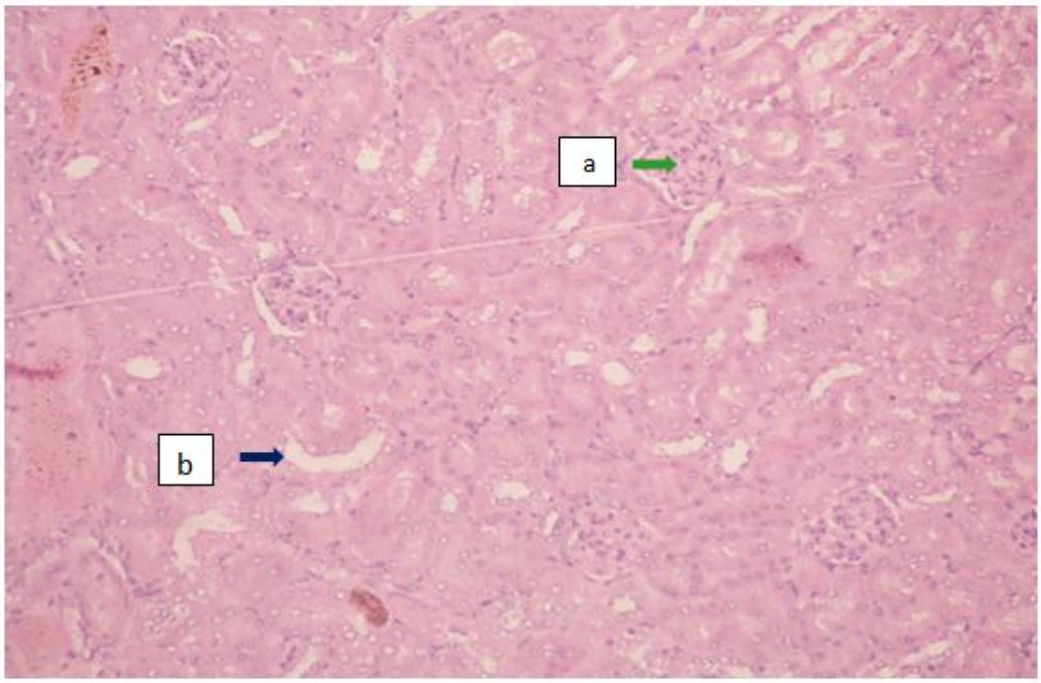


Fig. 8. Photomicrographs of kidney sections of mice injected with normal saline only showed:- (a) Normal renal glomeruli and (b) Normal renal tubules. (40x)

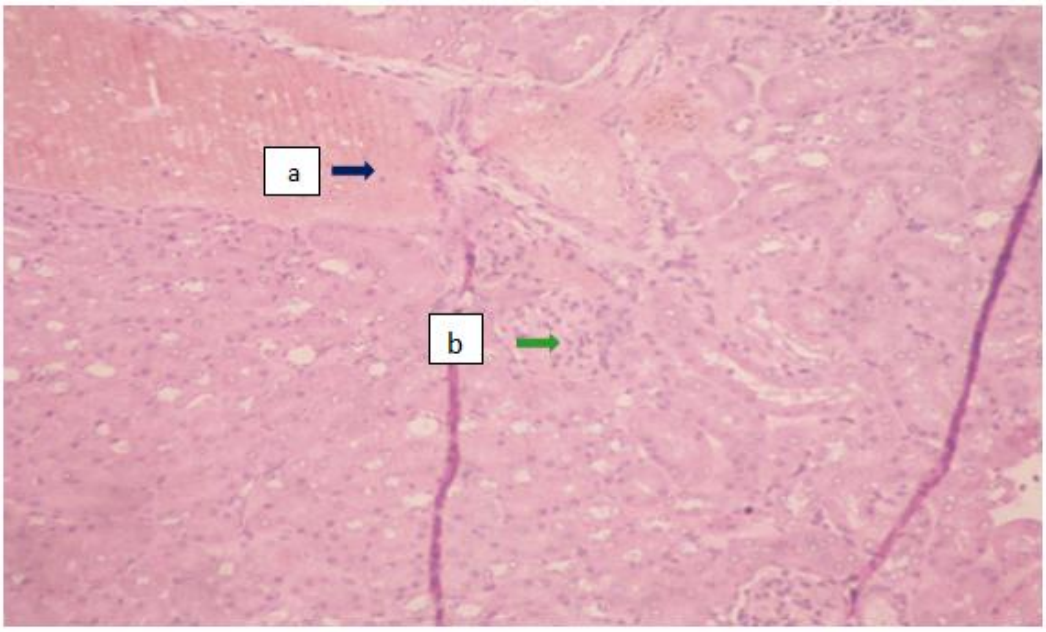


Fig. 9. Photomicrographs of kidney section of mice injected with EAC only, showed: - (a) Loss of architecture and (b) Mitotic figures. (40x)

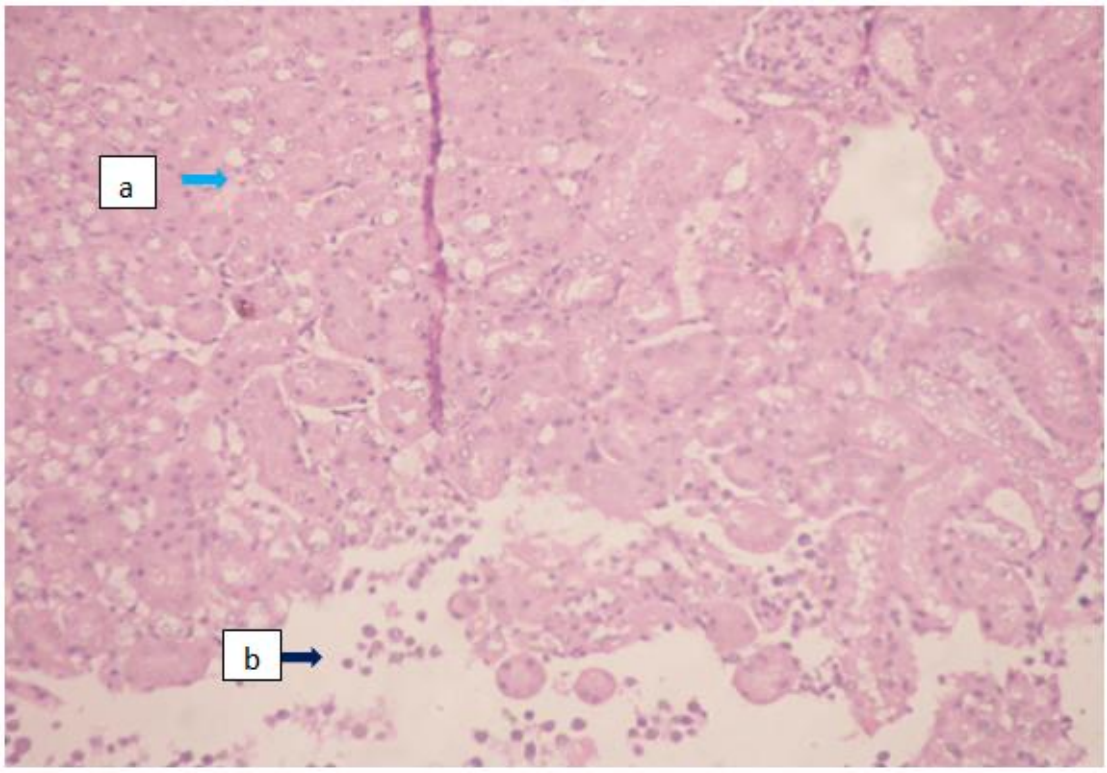


Fig. 10. Photomicrographs of kidney sections of mice injected with EAC+ TD2 showed:- (a) Regrets tumor mass and (b) apoptosis. (40x)

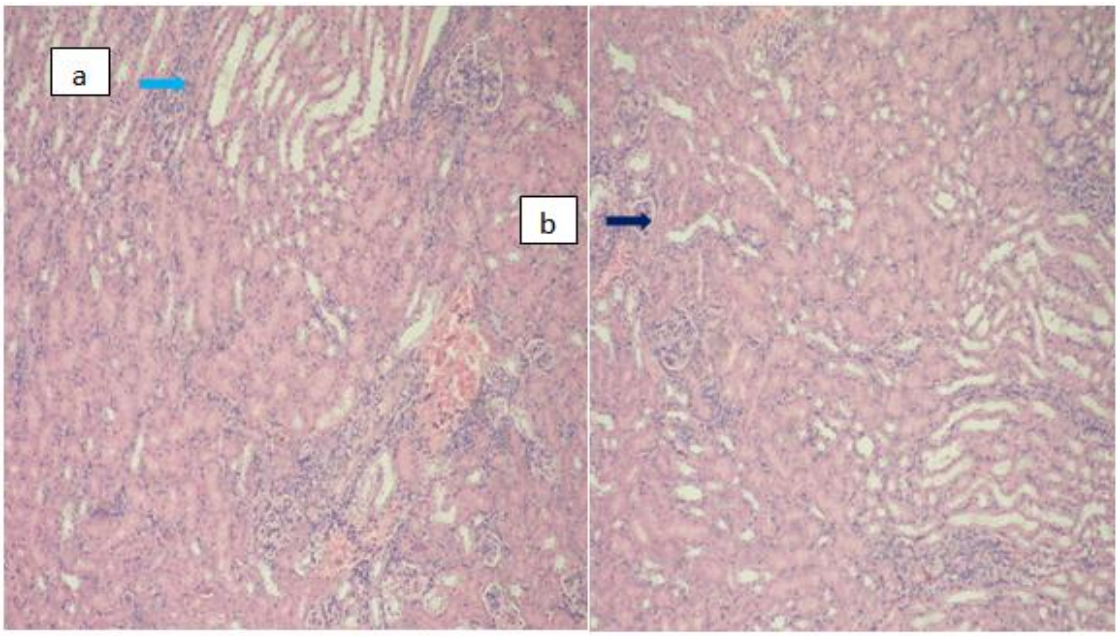


Fig. 11. Photomicrographs of kidney section of mice injected with (EAC+5 FU) showed:- (a) Infiltration in medulla and cortex and (b) congestion. (40x)

Table 5. The effect of TD2 on the oxidative status in liver tissues

	GSH (mg/g. tissue)	CAT (U/g. tissue)	SOD (U/g. tissue)	NO (µmol/ g. tissue)	MDA (nmol/ g. tissue)
saline	17.46 ±0.42	1.31 ±0.17	454.9 ±0.11	31.16 ±0.71	16.1 ±0.12
EAC	13.4 ±0.22	0.31 ±0.48	85.4 ±0.19	49.5 ±0.68	32.5 ±0.58
EAC+TD2	31.17** ±0.50	0.31* ±0.58	129* ±0.16	53.95*** ±0.11	13.87** ±0.74
EAC+5-FU	8.1*** ±0.96	0.63** ±0.78	345*** ±0.22	3.7*** ±0.13	24.3** ±0.02

*Values are represented as mean ±SE (n=7), Where * = Significant difference between test groups and EAC group,*P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001.where GSH: Reduced glutathione, SOD: Superoxide dismutase, NO: Nitric oxide, MDA: Malondialdehyde*

Table 6. The percentage of both aneuploid and diploid cells in different groups

Groups / Phase	EAC	EAC+TD2	EA+5-FU
Diploid G0/G1	57.3	74.1	63.8
Aneuploid G0/G1	31.6	19.9	21.6

3.10 Result of Cell Cycle Analysis

The percent of aneuploid cells in EAC control group increased compared to TD2 treated groups, while the percent of diploid cells in TD2 treated groups increased compared to EAC control group as shown in Table 6.

3.11 The Effect of TD2 on the Expression of P_{53} in Ehrlich Cells

The expression of P_{53} in mice received TD2, increased by 94 % compared to EAC control group as shown in Fig. 12 (a,b,c).

4. DISCUSSION

In this study we try to prove the efficacy of new compound of thiazole derivative as anti-tumor

agents. Firstly we examine its efficacy towards human cancer cell lines and the experiment gave excellent IC_{50} results.

Secondly we examine the efficacy on EAC bearing mice through in vivo research which gave also excellent results which support the *in vitro* results.

EAC is appeared firstly as a spontaneous murine mammary adeno-carcinoma [8]. It can form ascetic tumor in mice, so it is used frequently to study the antitumor efficacy of many compounds .In the present experiment increase in body weight, ascitic tumor volume, tumor weight and tumor cell count were observed in EAC control. Treatment of EAC tumor bearing mice with TD2 at the dose 2.5 mg/kg significantly decreased the body weight, tumor volume, tumor weight, and viable tumor cell count and increased the non-viable tumor cell count.

These results indicate either a direct cytotoxic effect of compounds on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition.

In the present study TD2 significantly increases the life span of the EAC treated mice. Review of literature confirm that increase in life span of tumor bearing mice, decrease in tumor volume and viable cell count, and elevation of non-viable cell count is a evidences for the efficacy of any antitumor agents [9] and TD2 achieve this criterion.

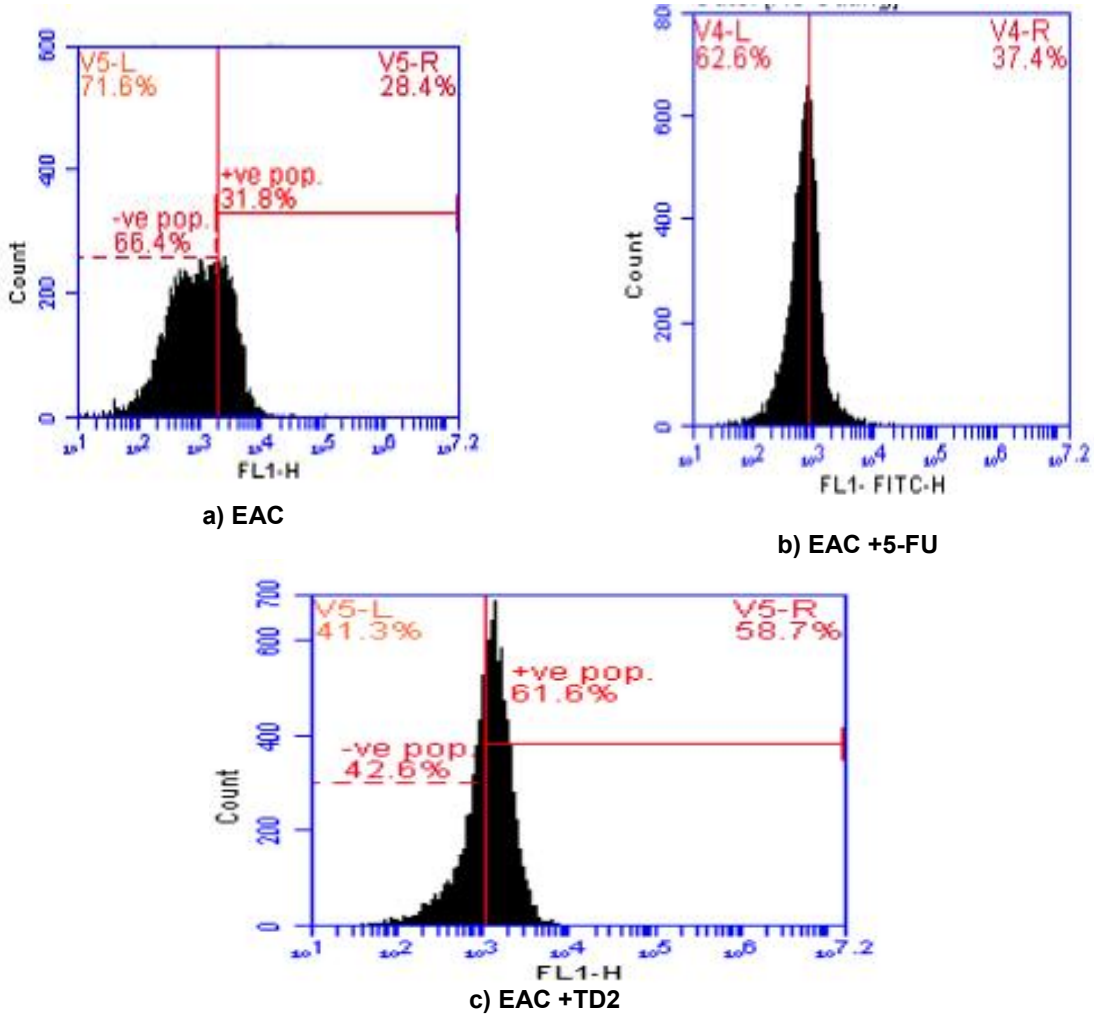


Fig. 12. Flow cytometric histogram shows the effect of TD2 and 5 -FU on the expression of P_{53} in EAC

The major problems encountered in cancer chemotherapy are myelo suppression and anemia, which are mainly because of iron deficiency, either due to haemolytic or myelopathic conditions finally leads to reduction in RBC count or hemoglobin content. Similarly, in the present study reduced hemoglobin, RBC count and WBC count was observed in EAC control group, RBCs count was increased in TD2 groups by 51% rcompared to EAC control group which supports its reduced myelotoxicity and increased hematopoietic protecting activity and free radical scavenging activity, while hemoglobin concentration was increased in TD2 group by 15% as compared to EAC control group. In addition, administration of TD2 raised WBCs count by 1000% compared to EAC control group.

Values of ALT (Alanine aminotransferase), AST (aspartate aminotransferase), and Albumin were elevated in the EAC control group but lowered in drug groups. TGL (triglyceraldehde) also was lowered in drug group.TD2 treatment also decreased the levels of MDA(malondialdehyde) and raised the values of GSH (reduced glutathione), Catalase and SOD (superoxide dismutase).

These results prove the cytotoxic efficacy of TD2 on tumor cells .Also in this research, a decrease in SOD, CAT and GSH and increase in MDA levels were noticed in EAC bearing mice. TD2 treatment prevent the decrease of antioxidant levels and rise in MDA levels in EAC bearing mice which prove the potency of TD2 in protecting against oxidative stress by normalization of antioxidants values in EAC bearing mice.

Lowering in antioxidants and raising in MDA is assign of cancer. Flow cytometric analysis shows that Arrest of cell cycle mostly occur in S phase then lead to apoptosis, because the aneuploid ratio is permanently and strongly relate with S phase which produce proliferation this may indicate that TD2 interfere with DNA replication.

The histopathology inspection of the liver & kidney of EAC control mice has shown many abnormalities in comparing with normal one ,as congested central vein, exfoliated epithelial cells, congested sinusoids and mitotic figures, as a result of cytotoxicity of the tumor. Modulation of the above mentioned effects which have been noticed in TD2 treated mice proved the strong antioxidant and hepatoprotective effects of the compounds.

Flow cytometric examination of P_{53} showed increased percentage of the positive population in treated group than control group (approximately double increase) which supported the hypothesis of efficacy of TD2 as apoptosis inducer.

5. CONCLUSION

It was concluded that TD2 have a potent antitumor activity against Ehrlich ascites carcinoma in mice beside a cytotoxic effect on MCF-7, PC3, HepG2 and HCT-116 (human cancer cell lines).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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