

**SYNTHESIS AND ANTI-PROLIFERATIVE ACTIVITY OF SOME
ISOINDOLINE-1, 3-DIONE DERIVATIVES AGAINST EHRlich'S
ASCITES CARCINOMA BEARING MICE MODEL**

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Summary

Five (1a-5e) isoindoline-1, 3-dione derivatives were synthesized and characterized by IR, ¹H NMR, and Mass spectroscopy. They were evaluated for *in vivo* anticancer activity against the Ehrlich Ascites Carcinoma bearing mice model. Male Swiss albino mice were used as test animals. The synthesized (1a-5e) compounds were administered intraperitoneally at a dose of 20-25 mg/kg body wt. per day for seven days after 24 hrs of tumor inoculation in mice. The standard drug used was 5-Fluorouracil (20 mg/kg, b. wt.). Compounds treated (III-VII) groups were found to reduce the body weight, tumor volume, packed cell volume, viable cell count and increase the tumor weight (%) inhibition, ascites cells (%) inhibition and non-viable cell count and Increase in life span (% ILS). Compound 4d showed the highest inhibition of cancerous cell growth compared to other compounds. From the present study, it can be concluded that isoindoline-1, 3-dione derivatives might have potent anti-proliferative activity.

Keywords: Isoindoline-1, 3-dione derivatives; anticancer activity; tumor inhibition; Ascites cells inhibition.

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Introduction

Cancer is one of the most threatening diseases of the 20th century and spreading further with continuous and increasing incidence in 21st century [1]. Several methods exist for the treatment of cancer which includes chemotherapy, radiotherapy and surgery. Chemotherapy is now considered as the most effective method of cancer treatment. As most cancer chemotherapeutic agent severely affects the normal cells, further research is necessary to synthesize new bioactive molecules that must have new anticancer property, which cures cancer without damage to normal cells [2]. Isoindoline-1, 3-dione has been banned from the market since 1963 after it caused the worldwide teratogenic disaster. The babies exposed to isoindoline-1, 3-dione in utero during the first 34-50 days of pregnancy were born with severe life-threatening birth defects. Despite its unfortunate history, isoindoline-1, 3-dione has attracted scientific interest again because of its recently discovered action against inflammatory diseases and cancer. Its broad range of biological activities stem from its ability to moderate cytokine action in cancer and inflammatory diseases [3].

Isoindoline-1, 3-dione and its *N*-Substituted derivatives are well known to have a wide range of biological activities like anti-inflammatory [4], anticonvulsant [5], anticancer activity [5-7], and antimicrobial activity [8]. In this present study, we reported the synthesis of some five isoindoline-1, 3-dione-methyl/ethyl -aromatic acid derivatives (Fig.1) and evaluated their possible anticancer activity against Ehrlich Ascites Carcinoma (EAC) bearing mice model.

Chemistry

All chemicals used as analytical grade from Merck Chemical Co. (Germany). Melting points were determined by the digital melting point apparatus (Veego, VMP-DS) and are uncorrected. IR spectra were recorded on Perkin Elmer IR spectrophotometer (KBr disc) and ¹H NMR spectra on Bruker DRX300 NMR spectrometer (DMSO-d₆, CDCl₃, and TMS) and Mass Spectra.

Methodology

2-hydroxy-methyl-isoindoline-1, 3-dione

A mixture of isoindoline-1, 3-dione (0.1mole), formalin (0.25 mole), and anhydrous potassium carbonate (1g) was dissolved in water (50 ml) by heating on a heating mantle for 2 hrs. On cooling a solid separated, filtered, washed with water and recrystallized from ethanol as colorless needles⁸, m.p-140-141°C⁸, Chemical formula: C₉H₇O₃N, yield: 89.5%,¹HNMR (300 MHZ,

DMSO d_6 p p m): δ 6.38 (s 1H, OH), δ 4.95 (d, J= 6Hz, 2H, CH₂), δ 7.83-7.92 (m, 4H, J= 3Hz, ArH)

2-(2-hydroxy-ethyl-isoindoline-1, 3-dione)

Phthalic anhydride (0.12mol, 19.1gm) and amino ethanol (0.12mol, 7.32ml) were heated together in an oil bath at 100⁰C for two hours and diluted with HCl (50ml), recrystallized from ethanol (70%). Mp: 135⁰C, Chemical formula: C₁₀H₉O₃N, yield: 90.5%, ¹H NMR (300 MHz, DMSO d_6 p p m): δ 4.84 (s, 1H, OH), δ 3.57-3.63 (m, J= 3Hz, 2H, CH₂ CH₂), δ 7.82-7.83 (m, 4H, J= 3Hz, ArH).

3((1, 3-dioxoisindolin-2-yl) methyl/ethyl) Benzoic acid

A mixture of a 2-Hydroxy-methyl-isoindoline-1, 3-dione/ 2-(2-Hydroxy-ethyl-isoindoline-1, 3-dione) (0.2 mole) and aromatic carboxylic acid (0.2 mole) was dissolved in conc. H₂SO₄ (50 ml) by stirring at room temperature and subsequently poured into crushed ice. It was filtered, dried and recrystallized with acetone or ethanol.

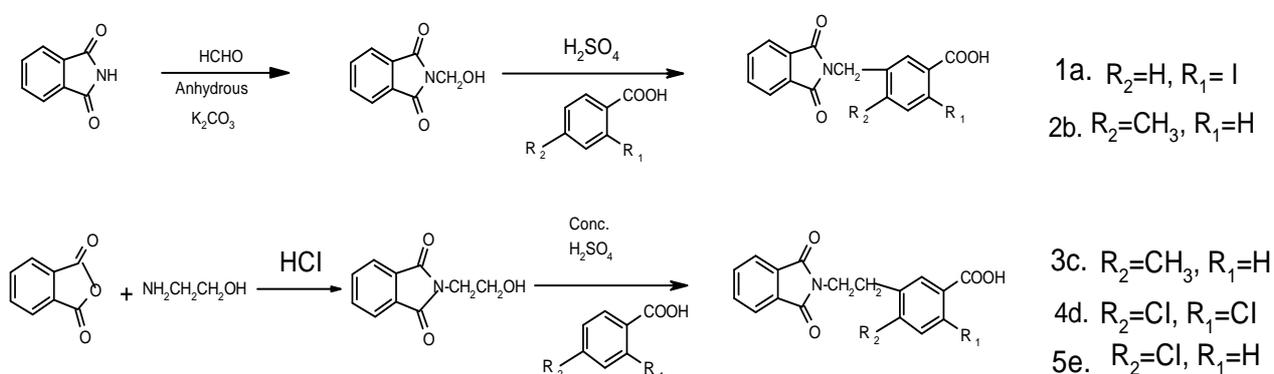


Figure 1. Synthetic pathway for compounds 1e–5e.

3((1, 3-dioxoisindolin-2-yl) methyl)-4-iodo-benzoic acid (1a)

Chemical formula: C₁₆H₁₀O₄IN. Yield: 88.5%. Melting point: 234-236^o C. IR (Vmax in cm⁻¹): 3456 (Ar-COOH), 2982 (ArC-H), 1390 (ArC=C), 11711(C=O) 1016, 1105, (N-CH₂), 528(ArC-I). ¹H NMR (300 MHz, DMSO d_6 ppm): δ 7.65 (s.1H, ArH), δ 7.93 (d, 1H, J= 6Hz, ArH), δ 7.18 (d, 1H, J= 6Hz, ArH), δ 7.89-7.86 (m, 4H, J=3 Hz, ArH), δ 4.76 (s, 2H, N-CH₂), δ 10.93 (s, 1H, ArCOOH). MSES+/-: 520.

3((1, 3-dioxoisindolin-2-yl) methyl)-4-methyl-benzoic acid (2b)

Chemical formula: C₁₇H₁₄O₄N. Yield: 84.75%. Melting point: 229-230 ° C. IR (Vmax in cm⁻¹): 3464 (ArCOOH), 3026, 2982 (ArC-H), 1714.4 (C=O), 1112.7 (N-CH₂), 1393.3(ArC=C). ¹HNMR (300 MHz, DMSO d₆ ppm): δ 7.76 (d, 1H, J= 6Hz, ArH), δ 7.31 (d, 1H, J=6Hz, ArH), δ 7.60 (s, 1H, ArH), δ 7.88 -7.90 (m, 4H, J =3Hz, ArH), δ 4.8 05 (s, 2H, N-CH₂), δ 2.46 (Ar-CH₃), δ 12.82 (s, 1H, ArCOOH). MSES+/-:297

3((1, 3-dioxoisindolin-2-yl) -ethyl)-4-methyl-benzoic acid (3c)

Chemical formula: C₁₈H₁₅O₄N. Yield: 60%. Melting point: 103-105° C. IR (Vmax in cm⁻¹): 3471 (ArCOOH), 3100, 2969 (ArC-H), 1405, 1310 (ArC=C), 1684 (C=O), 1270, 1085 (N-CH₂CH₂). ¹HNMR(300 MHz, DMSO d₆ ppm): δ 7.26 (d,1H, J= 6Hz, ArH), δ 7.42 (d, 1H, J= 6Hz, ArH), δ 7.44 (s, 1H, ArH), δ 7.85-7.82 (d,4H, J=3 Hz, ArH), δ 4.47-3.99 (m, J=3 Hz, 4H, N-CH₂CH₂), δ 2.51 (s, 3H, Ar-CH₃), δ 12.81 (s, 1H, ArCOOH) . MSES+/-:310.02

3((1, 3-dioxoisindolin-2-yl) ethyl)-2, 4-dichlorol-benzoic acid (4d)

Chemical formula: C₁₇H₁₁O₄Cl₂N. Yield: 65%. Melting point: 129-132 ° C. IR (Vmax in cm⁻¹): 3454 (ArCOOH), 3100, 2972 (ArC-H), 1391 (ArC=C), 1725 (C=O), 1101, 1047 (N-CH₂CH₂). ¹H NMR (300 MHz, DMSO d₆ ppm): δ 7.89-7.85 (m, 4H, J= 3 Hz, ArH), δ 7.79(s, 1H, ArH), δ 7.76 (s, 1H, ArH), δ 4.49-4.53 (m, J= 3Hz, 4H, N-CH₂CH₂), δ 12.82 (s, 1H, ArCOOH). MSES+/-: 365.06.

3((1, 3-dioxoisindolin-2-yl) ethyl)--4-chloro benzoic acid (5e)

Chemical formula: C₁₇H₁₂O₄ClN. Yield: 88.5%. Melting point: 220-221° C. IR (Vmax in cm⁻¹): 3469 (ArCOOH), 2966 (ArC-H), 1396 (ArC=C), 1777, 1687 (C=O), 1174, 1119, 1088 (N-CH₂CH₂). ¹HNMR (300 MHz, DMSO d₆ ppm): δ 8.32-8.14 (m, 4H, J= 3Hz, ArH), δ 7.94 (d, 1H, J= 6Hz, ArH), δ 7.56 (d, 1H, J= 6z, ArH), δ 4.76-3.97 (m, J=3Hz, N-CH₂CH₂), δ 13.19 (s, 1H, ArCOOH). MSES+/-:330.5

Materials and methods

Evaluation of Anticancer Potential

Animals

Animal studies were carried out using male Swiss albino mice of about eight weeks of age with an average body weight of 18-20 g were used for the experiment. The animals were obtained from Indian Institute of Chemical Biology (IICB), Kolkata, India. The animals were grouped and

housed in polyacrylic cages and maintained under standard laboratory conditions (temperature 30⁰ C) with dark and light cycle (12/12 h). They were fed a standard pellet diet and were given fresh water *ad libitum*. The mice were acclimatized to laboratory condition for 10 days before commencement of the experiment. All procedures described were reviewed and approved by the University Animals Ethical Committee (Registration No: 0367/01/C/CPCSEA), Jadavpur University, India.

Tumor cells

A tumor cells used for anticancer activity are EAC (Ehrlich Ascites Carcinoma) cells originated from human breast carcinoma. It is an undifferentiated tumor, which has lost its epithelial character. Ehrlich's ascites carcinoma (EAC) cells were obtained from Chittaranjan National Cancer Institute, Kolkata, India. The (EAC) cells were maintained *in vivo* in Swiss albino mice by intraperitoneal inoculation of 2 × 10⁶ cells/ mouse after 10 days. EAC cells of nine days old were used for the screening of the compounds.

Experimental procedure

Male Swiss albino mice of 8 weeks old with an average body weight of 18 to 20 g are used. All mice are kept on basal metabolic diet with water *ad libitum*. Male Swiss albino mice were divided into eight groups (n = 6). EAC cells are 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 these suspensions are counted under the microscope with the help of haemocytometer. All the groups were treated with EAC cells (0.2 ml of 2 × 10⁶ cells/mouse) intraperitoneally except the normal group. This was taken as day zero. In this instance, the tumor cells multiply relatively freely within the peritoneal cavity, and ascites develops. A day of incubation allows for establishing the disease in the body before starting the drug administration. On the first day, 5 ml/kg b.wt of normal saline (0.9 % NaCl w/v) was administered in group I (Normal). Normal saline (0.9 % w/v, NaCl), 5ml/kg, b. wt per day was administered in-group II (EAC control). The synthesized compounds (**1a**, **2b**, **3c**, **4d** and **5e** were administered at doses of 25, 25, 20, 20, 25 mg/kg, b.wt/day) and the standard drug 5-Fluorouracil (20 mg/kg, b.wt/day) were administered in groups (III-VII) and (VIII) respectively for 7 days orally at 24 h interval. Thus, 7 doses of drugs were administered to each mouse in the test group. On the 9th day food and water were withhold 18 hr before starting the testing operation. The weight of all the animals is recorded before they are sacrificed. The peritoneal cavity was dissected 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 was calculated. The fluid is sucked by adsorbent cotton. The weight of the 6 mice after sacrifice was recorded. 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: 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 Ascitic cells /ml in control animals. The anti-tumor activities of the compounds were measured in EAC animals with respect to the following parameters such as: Body weight, tumor weight, tumor cell count, tumor volume, viable and non-viable tumor cell count, mean survival time and percentage increase in life span, etc. [9-11]

Body weight

Body weight of the experimental mice was recorded both in the treated and control group at the beginning of the experiment (day 0) and on the final day prior sacrifice to evaluate the relative change. The body weight was significantly ($P < 0.001$) reduced when the EAC implanted animals were treated with compounds (1e–5e) and standard drug (5-Fluorouracil) for 7 days.

Tumor weight

The mice were dissected and the Ascitic fluid was collected from the peritoneal cavity. The tumor weight is calculated from the difference in weight of mice before dissection and after collection of Ascitic fluid after dissection. The tumor weight also significantly ($P < 0.001$) reduced when experimental group was compared with EAC induced group.

Tumor cell count

The Ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the numbers of cells in the 64 small squares were counted. The tumor cell count percentage inhibition significantly reduce ($P < 0.001$) when experimental group was compared with EAC induced group.

Tumor volume

The mice were dissected and the Ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000 g for 5 minutes, and it was also observed that the volume of EAC was significantly ($P < 0.001$) reduced when the EAC implanted animals was treated with compounds (1e–5e).

Viable and non viable tumor cell count

The cells were stained with trypan blue dye exclusion (0.9% in normal saline) dye assay. The cell that did not take up the dye was viable and those that took the stains were nonviable. The number of viable EAC cells was significantly ($P<0.005$) reduced, whereas the nonviable cells were found to be significantly ($P<0.005$) increased in drug and standard treated animal groups on the comparison with the EAC control animals.

Mean survival time and percentage increase in life span

Mean survival time (MST), and percentage increase in life span (% ILS) were calculated. MST of each group was monitored by recording the mortality for 6 weeks and % ILS was calculated using the following equation.

$MST = (\text{day of first death} - \text{Day of last death})/2$, $ILS (\%) = [(\text{Mean survival time of treated group} / \text{MST control Group}) - 1] \times 100$. An enhancement of life span by 20% or more was considered as the effective response. Treatment of synthesized compounds for the period of 7 days increased the survival time of the EAC-treated animals significantly ($P<0.01$) as shown in Table 2.

Tumor growth response

The anticancer activity of 1, 3, 4-Oxadiazole was assessed by observing change in the body weight, tumor weight, tumor cell count, Ascites tumor volume, packed cell volume, viable and non-viable tumor cell count, mean survival time (MST) and percentage increase in life span (% ILS). The mean time of each group of 6 mice was monitored by recording the mortality daily for 6 weeks and % ILS was calculated using equations [12-14].

Hematological studies

Hemoglobin (Hb) content, red blood cell (RBC) and white blood cell (WBC) count were measured from freely flowing retro orbital root blood. Differential WBC leukocyte count was carried out from Leishaman stained blood smears of normal, EAC control, and (1a-5e) treated groups respectively. (Hematological studies were done in Ashok's laboratory, Kolkata, India). The count of red blood cells and the content of hemoglobin, WBC, monocyte, neutrophil and lymphocyte were observed to be restored significantly ($P<0.001$) and ($P<0.005$) in all the drug-treated animals when compared with the EAC control.

Statistical analysis

Data were expressed as the Mean \pm S. E. M. The data were analyzed statistically using S. P. S. S Version 10. Software using ANOVA, Followed by Dunnet's multiple comparison test (DMRT). The minimum level of significance was fixed at $p < 0.001$.

Results and Discussion

The synthesized compounds (1a-5e) have significantly reduced the tumor weight and tumor cell count compared to that of the EAC control group as shown in Table 1. The tumor cell inhibition (% TCI) was 45.55 to 59.37% and the tumor weight inhibition (% TWI) is 32.30 to 65.23 %.

Table 1. Results of anticancer activity of the tested (1a-5e) compounds on % TWI and %TCI.

Group	Compounds	Avg tumor weight (g)	% TWI	Avg cell count (2×10^6)	%TCI
I	Normal Control	-	-	-	-
II	Induced control	3.25 ± 0.05	-	78.80 ± 0.74	-
III	1a	$2.10 \pm 0.02^*$	35.38	$38.85 \pm 0.01^*$	50.69
IV	2b	$2.20 \pm 0.01^*$	32.30	$42.09 \pm 0.11^*$	45.55
V	3c	$1.65 \pm 0.01^*$	49.23	$32.82 \pm 0.02^*$	58.35
VI	4d	$1.132 \pm 0.03^*$	65.23	$32.01 \pm 0.05^*$	59.37
VII	5e	$1.54 \pm 0.09^*$	52.61	$32.61 \pm 0.43^*$	58.61
VIII	Standard (5FU)	0.0 ± 0.01	99.98	00 ± 00	100

Value are Mean \pm SEM. n=6 animal in each group. Experimental groups were compared with Induce control; *P< 0.001.

According to the standards of National Cancer Institute, a substance is considered active if it exhibits the tumor growth inhibition by 50 %. All the tested compounds were found to show inhibition of tumor growth above 50 % (except compound 2b) which support the efficacy of the

Table 2. Results of anticancer activity of the tested (1a-5e) compounds(body weight, mean survival time, % ILS, tumor volume, packed volume, viable and non viable cell count of EAC bearing mice.)

parameter	Gr. II	Gr. III	Gr. IV	Gr. V	Gr. VI	Gr. VII	Gr. VIII
	EAC control (2×10^6 cell/ml per mice)	EAC + 1a	EAC + 2b	EAC + 3c	EAC + 4d	EAC + 5e	EAC + 5-FU
Body weight(g)	23.56 \pm 03	19.21 \pm 0.15***	19.20 \pm 0.03***	19.06 \pm 0.02***	18.22 \pm 0.21***	20.11 \pm 06***	18.17 \pm 0.07***
Mean survival time (days)	18.00 \pm 0.01	27.23 \pm 0.08***	21.71 \pm 0.11***	28.25 \pm 0.07***	32.37 \pm 0.08***	30.09 \pm 0.05***	41.14 \pm 0.05***
Increase in life span(% ILS)	-----	50.00	22.22	55.55	80.00	66.66	127.77
Tumor volume(ml)	2.05 \pm 0.03	1.25 \pm 0.02***	1.55 \pm 0.05***	1.01 \pm 0.01***	0.86 \pm 0.03 ***	0.88 \pm 0.02***	-----
Pack cell volume(ml)	1.25 \pm 07	0.96 \pm 0.01***	1.05 \pm 0.02***	0.89 \pm 0.02***	0.34 \pm .01***	0.58 \pm 0.01***	-----
Viable cell count($\times 10^7$ cells/ml)	73.17 \pm 0.03	17.05 \pm 0.36**	20.32 \pm 0.05**	10.11 \pm 0.37**	6.38 \pm 0.08 **	8.33 \pm 0.34**	-----
Nonviable cell count($\times 10^7$ cells/ml)	5.63 \pm 0.36	21.80 \pm 0.15**	21.77 \pm 0.08**	22.71 \pm 0.05**	25.36 \pm .08**	24.28 \pm 0.10**	-----

Value is Mean \pm SEM. n=6 animals in each group. ***P< 0.001, **P< 0.005 considered significant when experimental groups were compared with Induce control.

isoindoline-1, 3-dione derivatives to serve as potent anti cancer agents against EAC cells. The effect of compounds (1a-5e) on tumor volume, viable and non-viable cell count, and survival time was measured. (Table 2). The result showed significant reduction in tumor volume, packed cell volume, viable cell count and increase the non-viable cell count when compared to EAC control mice. The body weight reduces from 20.11 ± 06 to 18.22 ± 0.21 respectively of drug treated group.

The % of life span of animals was found to be 80.00 % to 22.22 %. However, the average life span of 5-FU treatment was found to be 127.77 %, showing its potent antitumor nature. The antitumor nature of isoindoline-1, 3-dione was evidenced by the significant reduction in the percent increase in body weight of animals treated with synthesized drugs when compared to EAC bearing mice. It was also supported by the significant reduction in packed cell volume and viable tumor cell count in synthesized (1a-5e) compound's treatment when compared to the EAC tumor control.

The hemoglobin content in the EAC control group was compared with experimental groups, an increased in percentage of hemoglobin in treated groups as compared to control group. Changes in RBC count were also observed in the treated groups, which may indicate that the synthesized isoindoline-1, 3-dione (1a-5e) derivatives shown significant antitumor activity against EAC cell lines bearing mice as compared to control group.

The total WBC counts were significantly higher in the EAC control group in comparison with normal mice. Whereas, the percentage of WBC count is significantly reduced in drug treated groups as compared to control group. The differential count, the percentage of neutrophil was increased in treated groups as compared to control group while the lymphocytes count was decreased in drug treated groups when compared with the EAC control group (Table 3). From the above study, the compounds may act as promising anticancer agents.

Table 3. Results of Hematological parameter of EAC bearing mice of the tested (1a-5e) compounds.

parameter	Group	Hemoglobin (g %)	RBC($\times 10^{12}/L$)	WBC($\times 10^9/L$)	Monocyte (%)	Neutrophil (%)	Lymphocyte (%)
Normal control (0.9% NaCl ml/mice)	I	12.05 \pm 0.28***	9.12 \pm 0.17***	4.23 \pm 0.07***	1.31 \pm 0.08***	16.05 \pm 0.21***	71.38 \pm 0.57**
EAC Control (2×10^6 cell/ml per mice)	II	4.49 \pm 0.12	2.26 \pm 0.07	21.17 \pm 0.09	1.03 \pm 0.01	81.09 \pm 0.03	21.39 \pm 0.06
EAC + 1a	III	8.60 \pm 0.13***	4.58 \pm 0.17***	11.97 \pm 0.22***	1.29 \pm 0.05***	42.68 \pm 0.24***	38.88 \pm 0.34**
EAC + 2b	IV	6.64 \pm 0.15***	4.29 \pm 0.07***	10.20 \pm 0.07***	1.13 \pm 0.04***	50.04 \pm 0.68***	22.98 \pm 0.30**
EAC + 3c	V	8.47 \pm 0.06***	5.47 \pm 0.11***	12.90 \pm 0.39***	1.26 \pm 0.01***	37.18 \pm 0.04***	39.86 \pm 0.32**
EAC + 4d	VI	10.51 \pm 0.15***	6.66 \pm 0.12***	16.07 \pm 0.20***	1.23 \pm 0.00***	27.36 \pm 0.14***	48.71 \pm 0.17**
EAC + 5e	VII	10.41 \pm 0.15***	5.61 \pm 0.12***	15.23 \pm 0.05***	1.25 \pm 0.01***	36.53 \pm 0.15***	41.12 \pm 0.43**

Value is Mean \pm SEM. n=6 animals in each group. ***P< 0.001, **P< 0.005 considered significant when experimental groups were compared with Induce control.

The exact mechanism of action of isoindoline-1, 3-dione derivatives is unknown. It may act due to multiple in events or apoptosis inducer. The compounds **4d**, **5e** showed significant anticancer activity. It may be due to chloro-phenyl ring attached to the isoindoline-1, 3-dione with ethyl groups respectively or the compound **4d** at 2, 4 positions *di*-chloro-substitution and in **5e** compound chloro-substitution at 4 positions of phenyl ring and tumor cell inhibition (%TCI) and the % of life span of animals was found respectively 59.37%, 58.61% and 80.00%, 66.66%. From the structural point of view, the chloro group which has the electron withdrawing property may be the crucial for tumor weight inhibition and tumor cell inhibition.

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